

THE EFFECTS OF DIFFERENT CONCENTRATIONS OF PHOSPHITE ON
ECTOMYCORRHIZA FORMATION BY *PISOLITHUS TINCTORIUS*
IN *CASTANEA DENTATA*.

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ABSTRACT

Phosphites, salts of phosphorous acid, are used to combat the devastating root-rot diseases of American chestnut (*Castanea dentata*) and other woody plants caused by the Oomycete *Phytophthora cinnamomi*. However, phosphite treatments may negatively affect the plant's ability to form ectomycorrhizas, the mutualistic symbioses between tree roots and certain soil fungi, and the phosphites themselves may be phytotoxic at high doses. Container-grown chestnut seedlings were treated with six phosphite solutions ranging from 0.0g/l to 12g/l both with and without a spore inoculation of the ectomycorrhizal fungus *Pisolithus tinctorius*. Seedling survival, height, root collar diameter, and ectomycorrhizal colonization were measured during one growing season. Spore inoculation significantly improved seedling survival across all phosphite doses. Phosphite applications of 1.5 g/l (the lower boundary of the manufacturer's recommendation) enhanced seedling growth and did not limit mycorrhiza formation significantly. Trees treated with highest doses of phosphite had lower survival, fewer mycorrhizas, and were significantly smaller.

DEDICATION

This manuscript is dedicated to the memories of Geraldine Jorgensen and Milton Longway, my grandparents, and my great uncle, Jimmy Youngberg.

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CHAPTER I

INTRODUCTION

Review of Chestnut

The American Chestnut (*Castanea dentata*) was described by Humphry Marshall in 1785 in his book, Arbustrum Americanum: the American Grove (Marshall, 1785). Marshall documented the known native species in North America at the time and gave descriptions and uses of trees and shrubs. He describes chestnut trees as “60-80 feet tall, 4-5 feet in diameter, mostly branch-free with long-toothed leaves.” He further informs the reader that in 1785 the tree was already used for railroad timber and was known to have greater durability (rot resistance) than oak. The tree’s nuts were used for substitute coffee and blacksmiths liked the wood as a heat source.

1891-1906 Exploring why *Castanea dentata* is important

The next publication, in 1891, the Pennsylvania State College Agricultural Experiment Station published Bulletin No. 16, which contained an article on the American chestnut (Buckhout and Frear, 1891). The article described cultivation of chestnuts as a nut crop. From 1891-1906, the years before discovery of Chestnut Blight, literature on the American chestnut tree focused on a variety of topics that ranged from pest management to medicine. The next few paragraphs explain the benefits and uses of chestnut - before blight - to demonstrate the importance of American chestnut.

Parks and Recreation

Prior to its elimination by the chestnut blight pandemic, the American chestnut was a much-appreciated species in what today is known as the parks and recreation field. American Chestnut was described as a “good large tree for creating shady spaces in the park” (Park and Commission, 1897). In the pre-blight literature, *Castanea dentata* was listed in a commissioner’s report for Wilmington Delaware as a species present in their park (Commissioners, 1898). It was listed as one of the few remaining species that was not sacrificed in Roger Williams Park for the purpose of citizen enjoyment (Southwick, 1903). It was even maintained inside the city limits of Washington D.C. (Fernow and Sudworth, 1891). The presence of the tree in the capital city indicates both its commonness and importance. A less-important tree might have been cut down to allow a more preferable tree to be planted or a sports field installed.

American Chestnut Distribution

During the pre-blight era, American chestnut had an expansive distribution across the eastern border of North America. One book, The Silva of North America: a description of the trees which grow naturally in North America exclusive of Mexico, indicated its range as Mississippi to Ontario to the Coast (Sargent, 1896). It was found in places such as all of Maine, Vermont (southeastern, with potential to cultivate statewide), southern Ontario, Lake Ontario shoreline, southeast Michigan and Michigan State Agricultural College (East Lansing), Massachusetts, Ohio (northeastern), Delaware, the Hudson Highlands in New York, New Jersey in the state and at the agricultural station, southeast Indiana, the Allegheny Mountains, Kentucky (Bell and Harlan counties), Tennessee, Illinois (Pulaski county), Alabama, Pennsylvania and Mississippi. In all

these areas, the common name was chestnut. (Beal, 1901, 1904; Berry, 1902; Clark et al., 1899; Earle, 1902; Education, 1901; Foley, 1903; Gleason, 1904; Hollick, 1900; Kearney, 1893; Kellerman, 1899; Macoun and Johnson, 1904; Mearns, 1898; Peck and Merrill, 1904; Society, 1900; Sudworth, 1898; Townsend, 1905). The native Americans of New York referred to the tree as O-heh-yah-tah, which roughly translated to prickly bur (Sudworth, 1898). One source identifies the tree as being present as far south as “west Florida and northward” (the original source was not obtainable for examination). In 1900, when experts examined this source, they proposed two potential reasons why it was no longer found in west Florida: 1) the species is migrating north and losing some of its southern territory, 2) The observation was recorded in error (Harper, 1900). In scientific literature, trees are known to migrate in response to environmental stressors. For instance, during climate changes, species might move northward and/or to higher elevations. This migratory action can be examined in many scientific fields, though most predominantly when looking at macrofossil and fossil pollen records (McLachlan and Clark, 2004; Woodall et al., 2009). Records provide an outline of the land area where *Castanea dentata* was autochthonous, more than 4400 square miles or 2.816 million acres, calculated as western Florida to Ball county Kentucky to southern Ontario to Maine to D.C and back to Northern Florida. Finally, *C. dentate* was recorded by two separate individuals to exist in two counties in Missouri. However, it was also indicated that these observations were likely misidentifications of *Castanea pumila* (Bush, 1895).

Experimental Plantings Outside the Native Range

The U.S. department of agriculture conducted experimental plantings of trees in the plains of Kansas, Nebraska, Colorado, South Dakota, Utah and Minnesota (Keffer, 1898). In these

experiments it was determined that American chestnut could be potentially grown in Kansas and Nebraska. Chestnut was grouped into a medium-shade group with good tolerance during youth which would benefit from protection during the decade following planting (Keffer, 1898). It seems likely that these experiments were for the purpose of assessing future forest crop cultivation plans in these areas. American Chestnut was planted in Iowa, though it did not occur there naturally, yet seemed to thrive (Fitzpatrick and Fitzpatrick, 1901). A single American Chestnut was noted at Eagle Lake in Indiana and was described as large and old but not likely to be native (Laboratory, 1902). In 1904, a publication which looked at various trees and their cold-weather hardiness in Manitoba and The North-West Territories, found that *Castanea dentata* died during the winter and was not compatible with such cold climates (Farms and Canada, 1904).

Botanical and Horticultural

The 1891 article which mentioned American chestnuts is titled The Transactions of the Linnean Society of London 2nd series: Botany: An enumeration of all the Species of Musci and Hepaticae recorded from Japan (Mitten, 1891). American Chestnut was again described in 1901, with the most-detailed description up to that time (Dame and Brooks, 1901). American Chestnut is self-sterile, as evidenced by the lack of nut production in fields with only one tree, and requires good soils for growth (Gifford, 1897; Parry, 1897). This growth pattern indicates that for a thriving population, a certain minimum density needs to exist to create a viable tree population. The largest recorded American Chestnut in Ohio by 1903 was 13'3" in circumference 3-4' above the ground (Club and Science, 1903). This is smaller than a tree mentioned in an earlier article, which noted that the trees could grow to a size of 120' tall and 13' in diameter, were very common and shade intolerant (Pinchot and Ashe, 1897). Chestnut trees were so common that even the Teachers'

Manual for Second Reader contained a description and some quotes from books such as Nature Study in Elementary Schools (Funk and Moses, 1904).

Research in 1903 examined the effects of altitude on the upper limit for the existence of *Castanea dentata* (Harshberger, 1903), revealing that the upper limit was approximately 5,200'. The researchers observed edaphic control of chestnut location related to the location of streams (Harshberger, 1903). There is likely a hard ceiling to the altitude limits of the species due to shallow atmosphere.

In Minnesota during 1903, a one-year-old chestnut seedling could be expected to be 6-12 inches tall when planted in good to average soils (Green, 1903). For comparative value, at the University of Tennessee at Chattanooga nursery, an American chestnut tree might grow trees 6' in the first year in a potted system utilizing a greenhouse, proper fertilization, mycorrhizal inoculation, watering and plenty of sunlight. Pinchot and Ashe observed that the American chestnut trees required good access to light (Pinchot and Ashe, 1897).

The flora on two summits (King's Mountain and Crowder's Mountain) in North Carolina were found to have dwarfed vegetation. American Chestnut adults at the two locations ranged from 3-6' in height and produced a large quantity of nuts (Small, 1901). It would be interesting to visit these locations to search for any remnants of the American chestnut species to determine if these trees had different genetics from the larger population, or experienced dwarfism due to environmental constraints. Location of individuals on these two mountains could lead to the acquisition of valuable genetics for the purpose of nut crops.

Medical

Researching a tree that was practically eliminated from the environment 100 years ago brought a surprise. The species was important medically. Many of the references to medicine were listings in the US Pharmacopæia, listing the plant name and dosing information. (Rusby, 1892). For some books, the pdf file was incomplete for the part relevant to American chestnut. This resulted in examination of a list indicating that there was further information available (Millspaugh, 1892). In 1894, the book An Aid to materia medica, *Castanea dentata* was to be used by taking the leaves and extracting fluid from the leaf to get 3.75-7.50 cc of fluid extract. Unfortunately there was no indication of what the extract was used to treat (Dawbarn, 1894). A separate source in the same year indicated that one of the medically interesting compounds were tannins (Gray, 1894). In 1895, the extraction process was detailed more thoroughly as: the fluid extract from *Castanea* leaves, which is a concoction of glycerin, alcohol and a percolated extract from crushed leaves (Harrop, 1895). The 1895 literature indicated that the extract could be used as a mild sedative and the medically interesting constituents were tannin, gum, albumin and resin and 2-8 grams of extract was sufficient (Wright, 1895). In 1896, the pharmacopoeia was updated, but the chestnut information remained the same (Wright, 1896). By 1904 the medical evolution of chestnut leaf fluid extract was used primarily as an antispasmodic (muscle spasm suppressant) for the treatment of whooping cough (Nelson and Co, 1904). It was considered to be well understood chemically but more physiological research was needed (Wright, 1905). If current restoration efforts go well, there might be some benefit from examination by the pharmaceutical industry of the American chestnut.

Etymology

The word “*dentata*”, like dentists, dental and other similar words refers to the serrated edge of the leaf which appears toothed (Huntington, 1902). There are two competing ideas regarding the etymology of “*Castanea*”. One article suggests that the etymology of *Castanea* was derived from a city located in Pontus (Gray, 1894). A second source indicates that the name *Castanea* comes from a town in Thessaly (Huntington, 1902). No conclusive answer for the etymology was located during review of literature.

Phylogeny

In 1892 there was debate about the scientific name for American chestnut. The author, Geo. B. Sudworth, Forestry Division, USDA conclusively finished on the note that indeed the correct name for the American chestnut should be *Fagus Castanea dentata*, separating it from the European and Asian chestnuts (Sudworth, 1892). In an 1895 publication, titled Ohio Fungi, there is a proposal to change the name of *Castanea sativa var. americana* to *Castanea dentata* (Kellerman, 1895). A brief search of the literature on “*Castanea sativa var. americana*” revealed 21 publications ranging from 1891-1916 and one instance in 2014. These publications contain similar material to the literature located under *Castanea dentata* (some of the articles are present in both groups). Curiously, none of the publications using the name ‘*Castanea sativa var. americana*’ are referenced in the medical literature. Instead, these publications are limited to botanical topics while the medical literature exclusively utilized the name *C. dentata*.

Economic

Prior to the chestnut blight, chestnuts had very high prevalence. In fact the author of Familiar Trees and their Leaves, the author F. S. Matthews describes the American chestnut as “so familiar to everyone who lives in or near one of our great cities, in whose vicinity it is pretty sure to be planted, that a description of the tree seems wholly unnecessary for its identification.” (Mathews, 1896). Familiar Trees and their Leaves outlines some of the many economic benefits of the species: an extraordinary cash crop, rapidly fruiting with marketable product and rapidly maturing to timber stage and finally the coppice growing to timber stage even more rapidly (Mathews, 1896).

The economic potential was outlined in great detail in an 1895 Pennsylvania Forestry Report, which advised that farming of chestnut should occur for five reasons (Wirt, 1902).:

- 1) It will grow almost anywhere (Wirt, 1902).
- 2) It grows quickly (Wirt, 1902).
- 3) It rapidly produces a coppice crop (Wirt, 1902).
- 4) It is desirable for the lumber and the fruit (Wirt, 1902).
- 5) Demand will increase because of the tannin content (Wirt, 1902).

Apiculture

The American chestnut was important in the apiculture (large scale beekeeping) and thus some research was conducted on important times for collection of American chestnut honey (above 40° North is June-July, and below 35° North is April-May) (Benton, 1896). This can provide

insightful information about the approximate bloom and pollination times in the original population of American chestnut tree.

Lumber (Timber), Coppice and Forestry

A book, The white pine: a study, with tables of volume and yield, which focuses on white pine, examines the growth rates of various trees. According to a graph in the book, Chestnut outgrows white pine, ironwood and beech for the first 40 years. This indicates that if chestnut can be reestablished as a forest species, it might become more common and useful for lumber than white pine is currently (Pinchot and Graves, 1896). A table in this book examines tree heights of 17 different forest species at 10, 20, 30, 40 and 50 years. It shows that only the Tulip Tree grew taller in the first 30 years. At 40 years, white pine, white ash and tulip tree were all taller and after 50 years Norway pine, red oak, black birch and basswood all grew taller than chestnut (Pinchot and Graves, 1896). The idea of replacing our white pine timber forests with chestnut forests for lumber production seems quite appealing; the advantage of quick growth during normal forestry rotation time periods (30 years) indicates that more board feet might be obtainable from the same number of plants. American chestnut could be cross-planted with alternate rows of white pine or corn to make the transition to chestnut crop less financially burdensome (Wirt, 1902). This process could help with monocrop concerns for individual farmers, since acreage gets multiple uses when using mixed- crop farming. An additional forestry technique of dense planting and removing individuals as the stand matures can potentially help farmers bring in income while establishing their chestnut orchards for lumber, coppice or nut crop farming.

In the southern Appalachians the tree was very valuable and had more rapid growth than any other native hardwood (Ayres and Ashe, 1905). Ayres and Ashe certainly add to the weight of evidence that *Castanea dentata* should be examined for its potential to replace some of the white pine production in today's economy. According to Pinchot and Ashe (1897), American chestnut was a valuable coppice crop (Pinchot and Ashe, 1897). Coppicing is regrowth that occurs after the tree is cut down. Coppice capability helped preserve the genome of the American chestnut after the blight infected the population. Fortunately, the blight does not infect the root system so the infected trees can still coppice and potentially reproduce.

Properties & Uses of Lumber and Coppice

The book The Silva of North America: a description of the trees which grow naturally in North America exclusive of Mexico was a good resource for specific parameters of the wood density and other physical parameters of the pre-blight population (Sargent, 1896). This book mentions some of the uses of chestnut wood, which include: "cheap furniture, interior home finishing, railroad ties and fence posts" (Sargent, 1896). The book mentions what appeared at the time to be the first mention of the American chestnut in a narrative published in 1609 (Sargent, 1896). This 1609 reference might possibly be the earliest written record of American chestnut in English. American chestnuts had very desirable wood lumber properties, including "light", "coarse-grained" and "easily split". These properties resulted in its use in many industries such as "cabinet-making", "railway ties" and "fencing"(Pinchot and Ashe, 1897).

Chestnut Nuts

The nut crop itself was known to be nutritious and delicious and as a result, it was a lucrative crop. The chestnut was praised as being the premier choice for many reasons: number of trees that can be planted, fruit quality and fruit quantity (Parry, 1897). A chestnut orchard can bring better returns than a farmer's field of the same space. In an uncited French study, chestnut yields 16% sugar (Huntington, 1902).

Additional Economic Benefits

The American chestnut had several other properties, such as high tannin content and medicinal uses (Pinchot and Ashe, 1897). In a rather interesting article, archeologists were excavating Baum Village and found grains and seeds inside pits and with these seeds were found the shells of American Chestnuts (Mills, 1901). This finding demonstrates the importance of the American chestnut to people (Native Americans) well before written history. This study presents further evidence that the tree was economically important before even being considered for lumber.

In 1905, the year before chestnut blight was discovered, a wonderful text about chestnut culture was published (Sterling, 1905). The book, Chestnut culture in northeastern United States, covers topics ranging from the history of the American, European, and Japanese chestnuts (Sterling, 1905). It explores the subjects of etymology, grafting, named cultivars, and uses for American Chestnuts (Sterling, 1905). A list of the uses includes: Food (flour for bread, fresh, roasted, steamed pureed), Nutrition (similar to wheat but more can be harvested annually from the

same acreage, and cultivation requires much less work), Coppice (20-30 year rotations), Lumber (30-50 trees per acre 70-100 year rotations) (Sterling, 1905).

Pests and Ecological Relations

In the pre-blight literature, there are a few sources which mention some ecological and or pest-related issues. In Ohio they found that San Jose scale was a pest which attacked American Chestnut and the scale was a terrible problem for many tree species and for many nurseries (Webster, 1897). American Chestnut is a host species for insects in the Aphididae family in North America (Hunter, 1901). American Chestnut was found to be an important food for crow blackbirds as discovered by stomach content analysis in 1900 (Beal, 1900). This study looked at only three species of birds for stomach content analysis. It would be expected that other bird species, small mammals, possibly even large mammals relied on chestnuts as a carbohydrate source to build up body fat and survive the winter. Following the blight, the ecological role of providing food was filled by other species, including the smaller and much less tasty oak nuts (acorns). In 1904 scientists began examining which fungi had relationships with trees and found that *Septoria ochroleuca* was examined on *C. dentata* (Kellerman, 1904). This was the earliest literature found which examined fungi and chestnut relationships. A terrible pest, the chestnut weevil is identified just prior to the blight (Sterling, 1905).

Roots

Ectomycorrhizae are present on roots, so it is only natural that the pre-blight literature on roots be considered. Possibly the first drawing of the roots of the American Chestnut ever

published were of a seedling comparing the Amentiferae plant family (Rourlee and Hastings, 1898). Rhizomorphic root-rot was observed on fruit trees including *Castanea dentata* (Wilcox, 1901). The year 1903 saw research into etiolation of chestnut, the study of growth of the seedling in the dark, giving insight into the processes which occur under the soil as a new seedling seeks sunlight (MacDougal, 1903). It was noted that chestnut trees form strong lateral roots (Pinchot and Ashe, 1897). These four articles were the only material on chestnut roots located in the literature search.

Chestnut Blight Discovered

In 1904, a new fungal and very lethal chestnut disease, *Diaporthe parasitica* was reported and proven by completion of a Koch's postulate experiment (Murrill, 1906). By 1906, the disease was already observed in Maryland, New York, New Jersey and Virginia. The assumption is that the disease arrived in America shortly before 1904, when the New York Botanical Garden began researching the disease (Murrill, 1906). By 1908, the disease had spread rather rapidly and caused several million dollars of damage in New York alone (Murrill, 1908). The origin of the disease and where it began in North America were unknown, as of Murrill's 1908 publication, in which Murrill portrayed the epidemic with a very fatalist tone, even suggesting that the disease could potentially wipe all *Castanea* species from the continent (Murrill, 1908).

Post-Blight

In 1917 Murrill's *Diaporthe parasitica* was determined to be *Endothia parasitica* (Shear et al., 1917). By 1919, *Endothia parasitica* was called its modern name of *Cryphonectria*

parasitica and was thought to have an insect vector, Cerambycidae, the long-horned beetle (Caesar and others, 1919). Ultimately, *Cryphonectria parasitica* killed the majority of the American Chestnuts in North America. The research previously had only been focused on lumber, nuts, tannins, economic benefits, farming and topics related to growth and use. Following the chestnut blight, research began to shift to more of a conservation theme. The conservation efforts ultimately lead to the creation of the American Chestnut Foundation.

The Backcross Breeding Program

The American Chestnut Foundation is devoted to the restoration of the American chestnut. The Foundation's plan for developing a blight-resistant American Chestnut is based on work begun in 1986 by Burnham et al. who published "Breeding blight-resistant chestnuts" (Burnham et al., 1986). They realized how important and vital to the eastern United States the chestnut had been prior to its decline. The early researchers realized the ecological importance of a species that had for thousands of years been a dominant food source for an entire ecosystem.

The backcross breeding program is essentially introgression of the genes for blight resistance from the Asian chestnut species into populations of the American chestnut. Blight-resistant Asian trees are bred with American chestnuts. The resulting interspecific F1 hybrids trees are grown for five years or so until they are large enough to be inoculated with the chestnut blight fungus. Screening for blight resistance is based on the size of the necrotic lesions, and on a suite of morphological characters to select for the American phenotype. The most highly resistant, American type trees are selected to advance to the next generation.

Selected F1 hybrid trees are crossed to American trees to produce the first backcross generation. These BC1 trees are, on average $\frac{3}{4}$ American. The screening, selection, and backcrossing steps can be repeated for two or three more generations, diluting the Asian portion of the genome by $\frac{1}{2}$ in every step, in such a way that the resulting genomes of the blight-resistant BC3 and BC4 trees are between 94% and 97% American. High levels of blight resistance, comparable to that of the Chinese and Japanese species, can be fixed in the hybrids by intercrossing selected BC4 tree for two or three more generations. The TACF ‘Restoration 1.0’ trees currently being evaluated, in collaboration with private land owners and with the US Forest Service, are BC3F3s that should be true-breeding for blight resistance. The goal is to reintroduce populations of blight-resistant trees that can survive and reproduce on their own under forest conditions, allowing for natural selection and evolution of the species to resume (Burhans et al., 2012; Craddock, 2006; Hebard, 2012; TACF, 2013).

Chestnut Pests

Castanea dentata has many current pests within orchards and the breeding program. Six of these (Polyphemus Moth (caterpillar), Ambrosia Beetle, Bagworm, Yellownecked Caterpillar, Japanese Beetle and Fire Ants) are noted as being present in modern research: Chestnut Cultivar Evaluations In Tennessee: Orchard Establishment and Early Germplasm Characterization (Craddock et al., 2005). The remaining four pests (Chestnut Weevil, *Phytophthora cinnamomi*, *Cryphonectria parasitica* and Gall Wasps) were observed in orchards while doing work that eventually led to this research.

Introduction/Discovery of *Phytophthora* in North America

Probably the best information regarding the early history of *Phytophthora* and its introduction into the range of the American Chestnut is in a 1945 article titled: Root disease of *Castanea* species and some coniferous and broadleaf nursery stocks, caused by *Phytophthora cinnamomi*. Through literature review, the researchers determined that *P. cinnamomi* was introduced to North America, and impacted American chestnuts during the early 1800s (Crandall et al., 1945). The researchers further determined that the disease was probably imported from Asia by wealthy Americans importing plants for their estates (Crandall et al., 1945).

Phytophthora cinnamomi

Phytophthora cinnamomi is a common invasive pathogen in many plant systems worldwide, which causes a disease called root-rot. Perhaps the best resource to learn about *P. cinnamomi* is a book by George A. Zentmyer titled *Phytophthora cinnamomi* and the Diseases it Causes (Zentmyer and others, 1980). Zentmyer reviews the subject by examining 600 papers on *P. cinnamomi* (a sixth of which he was a contributor himself). He is an American author from California who was interested in the pathogen due to its effects on the avocado. Zentmyer found that the pathogen was present in over 60 countries, and that in Australia alone it attacks more than 400 individual species and had a total host count exceeding 1000 species (Zentmyer and others, 1980). In 1993 the number of Australian plant species known to be threatened by phytophthora had increased to over 2000 (Wills, 1993). It is therefore not surprising that much of the research on *P. cinnamomi* comes from Australia.

The pathogen *P. cinnamomi* is an organism which produces zoospores which swim through the soil, allowing it to infect new plants. As a result, soils which are more moist are more conducive to root-rot, while dryer soils are suppressive in nature (Broadbent and Baker, 1974). The pathogen infects roots just before the root tip, and the presence of an ectomycorrhizae provide protection from infection (Barham et al., 1974; Marx, 1969, 1973; Marx and Davey, 1969a, 1969b; Marx and others, 1970). Additionally, it is proposed that when the physical structure of the ectomycorrhizae is damaged (such as by nematodes), the *P. cinnamomi* is able to utilize the damaged area and infect the roots (Barham et al., 1974). The pathogen acts by infecting the root with mycelia used to absorb carbohydrates from the plant. The mycelia cause physical damage to the plant. In American chestnut, this damage to the root is usually fatal. A thorough review of the disease biology literature relevant to *P. cinnamomi* relevant specifically to *C. dentata* was produced by Mollie Ellen Bowles (Bowles, 2006).

Treatment of *Phytophthora cinnamomi*

Until the mid-1990s, there were minimal tools available to combat *P. cinnamomi*, including: locate and mark infected areas, conduct activities which might spread the pathogen only during dry periods, restrict movement in and out of infected locations, prevent moisture in the soil of diseased areas from travelling to healthy sites, strict decontamination of everything that moves in and out of diseased areas, increase awareness and hygiene of professionals and the general public (Hardy et al., 2001b). In 1977, awareness was building, and traction was gaining ground for a new chemical approach to treating *P. cinnamomi*...phosphate.

Phosphite

The chemical phosphite is used to treat root-rot in many species and typically has a high variance in relation to the effects both within and among species. In Australia, it was found to be 100% effective at prevention in approximately 20% of species. In the remaining 80% of species, the chemical only slowed the spread of root-rot (Pilbeam et al., 2000). In many species, phosphite is effective at combating root-rot but more often than not, the treatment is not lethal to *P. cinnamomi* (Ali and Guest, 1998; Hardy et al., 2001a; Pilbeam et al., 2000; Wilkinson et al., 2001; Wills, 1993; Zentmyer and others, 1980).

Chemically, phosphite is anionic, but when referred to for treatment of plants it is usually one of several salts of phosphonic acid (Figure 1 below shows a chemical diagram of the more common Phosphate, Figure 2 shows Phosphite).

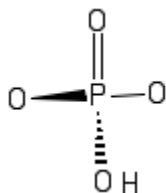


Figure 1. Phosphate

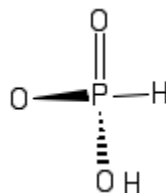


Figure 2. Phosphite

The major observation is that both molecules are tetrahedral in shape, but phosphite has one of its phosphate oxygen molecules replaced by a hydrogen molecule. This eliminates the mostly symmetrical charge located around the phosphorus atom. The result is that phosphite is much more chemically reactive and the phosphorous is much more available than in phosphate. A

good discussion on the believed chemical and biological mechanisms of phosphite, its slow transformation into phosphate, and the phytotoxicity, can be found in a 2001 article in the Journal of Plant Nutrition titled PHOSPHITE (PHOSPHOROUS ACID): ITS RELEVANCE IN THE ENVIRONMENT AND AGRICULTURE AND INFLUENCE ON PLANT PHOSPHATE STARVATION RESPONSE (McDonald et al., 2001). In the 1950s phosphite was determined to be a fertilizer, though more recent literature refutes this claim (Adams and CONRAD, 1953; Thao and Yamakawa, 2009). Regardless it has been marketed as fertilizer which allows easier distribution with fewer regulations.

Pisolithus tinctorius

Pisolithus tinctorius is an ectomycorrhizal fungus known in field trials to prevent colonization of root-rot (Barham et al., 1974). An ectomycorrhizal fungus is a fungus that forms a symbiotic relationship with the roots of a plant. To be considered an ectomycorrhizal relationship, the fungus must enclose the root with fungal material (mycelial sheath), must have hyphae which spread through the intercellular space (Hartig Net) and it must have hyphal filaments extending outward from the sheath to interface with the soil (Smith and Read, 1996). Ectomycorrhiza and plants have a symbiotic relationship, where the plants photosynthesize to produce sugars, which are then transported to the root system. At the root system, some of the energy source is given to the ectomycorrhizae. The ectomycorrhizae are able to utilize the hyphal ends to vastly improve the surface area of contact with the soil (Smith and Read, 1996). This increased surface area allows the fungus to absorb minerals and water from the surrounding medium at a much more effective rate than the roots could do alone (Smith and Read, 1996). The fungus returns the resources it doesn't use to the plant. In this fashion both the plant and the fungus benefit from the relationship.

Effects of Phosphite on Ectomycorrhizae

There are about 100 articles which focus on the effects of Phosphite on ectomycorrhizae, the majority from Australia. This research was initiated due to reports that phosphite was negatively affecting arbuscular mycorrhizae (Howard et al., 2000). The Australian teams have focused more on foliar spray application (possibly due to the large areas they are treating). They determined in their experiments that with foliar spray, *Pisolithus tinctorius* in eucalyptus was the most tolerant species of ectomycorrhizae to phosphite (Howard, 2001).

Phosphite was approved in 2003 in California for the treatment of Sudden Oak Death (*Phytophthora ramorum*). Shortly thereafter a surfactant was added, which allowed the chemical to penetrate the leaves, eliminating the need for injection application (Garbelott and Rizzo, 2005)

Effects of Phosphite on *Pisolithus tinctorius* in Chestnut

A poster presentation at Purdue University on phosphite and *P. tinctorius* (Zellers and Jacobs, date unknown) appears to have been planned to proceed during 2011, though no subsequent publication was located. In addition to this, the only other research specific to chestnut *P. tinctorius* and Phosphite is The Effect of Phosphite on Mycorrhiza Formation in American Chestnut (*Castanea Dentata*) (Perkins, 2012). His experiment examined three different ectomycorrhizal symbionts with *Castanea dentata* and their response to Phosphite. He found that there were differences in ectomycorrhizal growth between plants that received Phosphite treatment and those that did not. His project led directly into this current research, in which the questions are: “How do different concentrations of phosphite impact ectomycorrhizal growth in *Castanea*?” and “What dose of phosphite concentration with respect to plant and ectomycorrhizal growth is

ideal for *Castanea*?” The purpose of answering these questions is to determine current best-practices for handling root-rot while breeding for blight-resistance, and possibly even future breeding by the American Chestnut Foundation for phytophthora-resistance.

CHAPTER II

METHODS

Site

The study site consisted of the chestnut research greenhouse and nursery facility at the University of Tennessee at Chattanooga. The greenhouse is approximately 60' by 30' and has a capacity to produce about 1400 plants in 2-gallon containers. The entire nursery is approximately 120' x 120' and has drip irrigation capacity for 1600 2-gallon containers outdoors in addition to the glasshouse capacity.

Experimental Design

The experiment was conducted on 240 container-grown seedlings of a half-sibling family of hybrid American chestnut trees. The experimental trees were grown from open-pollinated seeds of TACF-SA333 collected from a grafted specimen at the Smith Farm Orchard. TACF-SA333 is a BC2F2 (an intercross of two selected second backcross trees) from the American Chestnut Foundation.

There were 12 treatments as described in Table 1. Each treatment included 20 seedlings. Half of the treatments were inoculated with spores of the ectomycorrhizal fungus *Pisolithus tinctorius*. The *P. tinctorius* spores were obtained from dried sporocarps collected locally in association with oak. Paired groups of inoculated and non-inoculated treatment groups received

phosphite doses in one of the following concentrations: 0, 0.75, 1.50, 3.00, 6.00, and 12.00 g/l. These concentrations were decided upon by consultation with the relevant literature and with Rick Fletcher of Cleary Chemical (Howard et al., 2000). The experiment began when the seeds were planted in the greenhouse on 15 May 2012. On 22 June 2012 the plants were moved from inside the greenhouse to the drip irrigation system outside.

Table 1. Experimental Design

		<i>P. tinctorius</i> spores applied in milligrams		
		69.281	0	Total
Phosphite application in grams per liter	0	Treatment A, n=20 (18)*	Treatment G, n=20 (14)	n=40 (32)
	0.75	Treatment B, n=20 (16)	Treatment H, n=20 (14)	n=40 (30)
	1.5	Treatment C, n=20 (16)	Treatment I, n=20 (13)	n=40 (29)
	3	Treatment D, n=20 (17)	Treatment J, n=20 (13)	n=40 (30)
	6	Treatment E, n=20 (17)	Treatment K, n=20 (13)	n=40 (30)
	12	Treatment F, n=20 (17)	Treatment L, n=20 (13)	n=40 (30)
	Total	n=120 (101)	n=120 (80)	N=240

*Each treatment began by planting 20 seed nuts. The actual number of trees at the start of phosphite treatment is in parentheses. The differences were due to poor seedling emergence and early seedling mortality.

Planting

The seeds were planted in new, 7.65 liter pots (Stuewe & Sons TP8162), containing a commercial, soilless potting medium (Sun Gro Horticulture Metro Mix SPM) composed of pine bark, sphagnum, and perlite. The containers were then fertilized with 1 tablespoon of Scott's Osmocote Pro (stock number 90170, 20-4-8), and then randomized and watered.

Watering

The plants in this experiment received the same watering methods normally used at this nursery. The first step, utilized inside the greenhouse, is to water each plant for a measured time allotment (usually three seconds) by hand as needed. Care is taken with this technique to ensure that each plant is watered as identically as possible to the other plants so that all plants remain on the same watering schedule. The second technique, utilized outside the greenhouse, is a drip irrigation system. Like the hand watering, the drip irrigation system is designed to provide the plants with as close to identical watering as possible to maintain the same watering schedule for all plants. There were four deviations throughout the experiment where these plants received a slightly modified water schedule from the remainder of the nursery. The first deviation was the initial watering at the time of planting, where the plants received watering and moisture levels needed for germination. The other three deviations occurred after each of three dose applications of phosphite. In each of these instances, the plants were given excess water before being removed from irrigation. Following watering the plants were removed from irrigation. Next, the plants were derandomized and height and/or root collar diameter measurements were recorded. The following

day, 500 ml of phosphite/water solution at their prescribed dose was given to each plant. The plants were then rerandomized and placed back on the irrigation system. For the following 48 hours they received no water, to allow the plants ample time to extract from the treatments as much phosphite chemical as possible.

Dosing

Two days after planting the seeds, the pots were divided into treatment groups A, B, C, D, E and F. The treatment groups were inoculated with the spores of *P. tinctorius*. The fruiting bodies were collected by Dr. J. Hill Craddock of the University of Tennessee at Chattanooga from under a local oak tree a few blocks away from campus. The spores were manually extracted by scraping into a container. The mass collected was determined to be 10.6 grams (dry weight) of spores, which was all that was available. This was compared to what was used in the previous study by Taylor Perkins (Perkins, 2012), who was consulted for the spore scraping and application. It is already established that 1 mg dry weight is adequate spores to establish a colony on a plant (Kendrick and others, 1985). Based on this, the 10.6 grams of spores is more than adequate for inoculation of 120 plants in this experiment.

The 10.6 grams of spores were mixed with 22.95 liters of new, unused potting medium and mixed by hand by two people for 1 hour to evenly distribute the spores throughout the potting medium. Mixing was conducted under positive airflow conditions to prevent any airborne spores from contaminating pots. The pots not receiving spores were additionally located further upwind than the ones which would be giving the spore/medium mixture. Additional care was taken to mix spores with medium in smaller quantities with small tools in order to minimize loss of spores to

the air. Mixing was conducted in two partially covered five gallon buckets. Final soil/spore mixtures from the buckets were combined and mixed further in a wheelbarrow to ensure consistency of product applied across the pots.

Then, 150 ml quantities of the spore suspension were taken and spread thinly and evenly across the surface of 120 pots. Similarly, the remaining 120 plants received 150 ml of new, unused potting medium without spores, spread thinly and evenly across their surface. After spore application, which occurred two days following initial seed planting, the pots were watered and randomized. After randomization, pots with/without spores were divided into the 12 treatment groups.

Spore Dose Calculation

The other factor, phosphite was mixed using standard lab equipment. The plants were given prescribed concentrations (see Table 2 on the next page). In Table 2, the first column is the experimental dose desired, the second column is the calculated quantity of Alude (Cleary Chemical brand phosphites) and column 3 is the measured quantity used on the plants in the designated treatment groups. Alude contains monopotassium phosphite (H_2KO_3P , CAS#13977-65-6) and dipotassium phosphite (HK_2O_3P , CAS#13492-26-7). It is 45.8% active ingredient by weight (5.17 lbs. of active ingredient per gallon and 3.35 lbs. Phosphorous per gallon).

Table 2. Conversion table for phosphite dose (g/l), calculated equivalent dose of Allude (ml Allude/l), and actual amount of Allude used (ml Allude/1.5 l)

Dose Strength	ml Allude/l (calculated)	Actual used (measured)
0.0 g/l	0.0 ml	0.00 ml / 1.5 l
0.75 g/l	1.20 ml	1.80 ml / 1.5 l
1.50 g/l	2.40 ml	3.60 ml / 1.5 l
3.00 g/l	4.81 ml	7.20 ml / 1.5 l
6.00 g/l	9.62 ml	14.40 ml / 1.5 l
12.00 g/l	19.23 ml	28.80 ml / 1.5 l

These concentrations were applied to the plants three times during the experiment: 9 June 2012, 21 July 2012, and 29 August 2012. When the time for the first dose arrived, I noticed that not all of the seeds had germinated. The number of seedlings in all treatments on 9 July is reported in Table 1 in parentheses.

Phosphite Application

When phosphite was applied, the plants were watered adequately the previous day, the plants were derandomized and then ordered by group (this was off of the drip irrigation system). The plants were then allowed to sit overnight. The next day the plants were administered phosphite,

by measuring with a beaker and then pouring directly at and around the base of the stem, 500 ml of the solution at the assigned concentration of phosphite as assigned according to Table 2.

Measurement of Seedling Height and Root Collar Diameter

Measurements included plant height in millimeters on three separate dates, and root collar diameter in 0.1 millimeter increments (the diameter of the main stem where it enters the potting medium). The times when plants were removed from randomization for phosphite treatments allowed brief opportunities to visualize differences which were occurring between groups. There are photos from one or two of these two day periods where differences appeared to exist from side by side visual observation of the groups.

On 8 August 2012, 30 September 2012 and 10 November 2012, seedling heights were measured in mm and each group's plants were averaged, with the means listed in Tables 4-6. On 30 September 2012 and 17 November 2012, the root collar diameter was measured and averaged per the groups listed in Tables 4-6. Then, a sample of root length was cut and labeled "length of root sample". The number of root branches off of the root was analyzed for tip(s) that possessed ectomycorrhizae. If a single tip had fungus, then the branch received a single count for the data "Root Branch with ecto". However, if no tips had fungus, that branch received a single count for "Root Branch without ecto". These data were summed and averaged to create the means in Tables 4-6. Averages were summed to create "total branch". The last three columns "Ecto per cm", "no ecto per cm" and "total per cm" were derived by dividing "Root branch with ecto", "Root branch without ecto" and "total branch" by "Length of root Sample".

Ectomycorrhizal Quantification

On 17 November 2012 all plants were removed from the potting medium to measure the root collar diameter. Then, the three secondary roots were collected nearest the surface at least 10 cm in length. If inadequate root was present, additional roots were collected and in some case the entire root system. These roots were then shaken lightly to removed potting medium and then wrapped in a paper towel and placed in Ziploc-brand freezer bags with 20 ml of 50% ethanol solution (to preserve the roots, until further work could be completed). Sample bags were stored under refrigeration at 4° C.

The actual quantification of mycorrhizas was a modified version of the procedure used by Perkins (Perkins, 2012) at the University of Tennessee at Chattanooga. In Perkins' research, roots were placed in water and viewed under a dissecting microscope. Tertiary roots were visually examined for the presence of ectomycorrhizae. Each tertiary root was counted that contained a root tip with ectomycorrhizae. The sum for each secondary root was divided by the length of the root to create a metric of root tips per cm.

In the current research, roots were removed from the ethanol storage and rinsed in water to remove any additional potting medium. Roots were then allowed to slightly air dry, allowing them to be dry enough to fan out but wet enough that ectomycorrhizae remained visible. If the plants were too dry counting was difficult and if too wet, the roots would clump. Once the roots were dried to an appropriate dampness, they were fanned out and scanned (with a ruler) in a high resolution digital flat-bed scanner. The images were analyzed using Adobe Photoshop, where the lengths of the secondary roots were measured and where root tips of tertiary roots were examined and counted.

When examining the root tips, a second modification to the previous research was implemented. This modification consisted of counting the number of non-ectomycorrhizal tertiary roots as well as the ectomycorrhizal ones. This allowed for statistical analysis of additional root properties, including root density per cm, proportion of ectomycorrhizal to non-ectomycorrhizal roots and of course, ectomycorrhizal and non-ectomycorrhizal roots per cm.

Root Lengths

The lengths of roots were measured by printing the scanned images (with rulers for scale measurement). Then string was placed along the root length to be measured and the beginning and ends of the quantified sections were marked. The string was then compared to the scale ruler and an exact length determined.

Analysis

Quantified data were analyzed utilizing ANOVA and Tukey's Honestly Significantly Difference tests (Zar and others, 1999). Assumptions for both tests were checked visually utilizing boxplots and scatter plots for the appropriate data sets. The software package R and the plugin multcomp were utilized for the statistical analysis, including assumption checking. An alpha level of 0.05 was utilized for all significance decisions made in the research and conclusions (Hothorn et al., 2014; Team and others, 2012). Only plants which survived the entire experiment were analyzed, due to the difficulty associated with correctly determining the correct cause of death.

Two-Factor Analysis of Variance

Analysis of Variance (ANOVA) is a collection of statistical models which can be utilized to determine difference between group means and their associated procedures such as “variation” among and between groups. In ANOVA models the total variance observed in a given variable is divided into parts, which may be sources of variation (example: variation among groups or variation between groups). In the most simplistic form, ANOVA allows an easy and effective way to determine if the means of various groups are equal or not. ANOVA is capable of generalizing the t-test and allow testing of multiple groups for statistically significant differences.

In this experiment the Null Hypothesis (the assumed correct answer) is:

$$\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 = \mu_6$$

The Alternate Hypothesis (what the test is checking for) is:

$$\textit{At least one } \mu_i \textit{ is different from at least one other } \mu_i$$

This analysis will use a classical two-factor ANOVA model

$$y = \alpha_i + \beta_j + \epsilon_{ij}$$

Where

$$\alpha_i = \textit{the effect of the } i\text{'th level of Factor A}$$

$$\beta_j = \textit{the effect of the } j\text{'th level of Factor B}$$

The ANOVA resulted in P-values that were used to test the significance of tested factors. In this experiment there were two factors: 1) Application of spores of an ectomycorrhizal fungus (yes/no), and 2) Amount of chemical phosphite applied at each of three treatment dates (0.0, 0.75,

1.5, 3.0, 6.0 and 12.0 grams of chemical phosphite per liter of water). The following collected data were analyzed in this method in two-factor ANOVA against Fungus, Phosphite, Fungus*Phosphite using weighted means. Traditionally in ANOVA it is mathematically desirable to have a balanced design (each group with the same number of data points) in order to maintain orthogonal data, which ensures that the groups being compared are equivalent and not biased. Unfortunately, in this experiment, some plants died for no apparent reason. There is difficulty in determining with certainty that the deaths were or were not a result of the treatments.

There are statistical methods available to analyze the data with or without the plants that died. In my research, the best results would be obtained by censoring (removing) the plants that did not successfully complete the experiment. In doing so the data set becomes unbalanced and the validity of a standard ANOVA comes into question. Weighting the means is a method of compensating for the gaps in unbalanced data by using means adjusted data. The calculations for the ANOVA data were produced using R, a software program useful for statistical analysis using the aov command and verified using the anova (lm) command using Type I Sum of Squares weighting. The following R code structure was utilized for the analysis:

```
aov(Response ~ Phosphite * Fungus)
```

Provided there was no significant interaction from the combination of Phosphite and Fungus factors, ANOVA was conducted with the following R structure:

```
aov(Response ~ Phosphite + Fungus)
```

All p-values can be viewed in Tables 8 & 9. Results with only two categories (such as fungus vs no fungus) need only be determined to be either significantly different or the same. The significant values in ANOVA tests with more than two possibilities can be further tested to

determine which groups within the factor are similar or different. A common statistical method for accomplishing this is called “Tukey’s Honestly Significant Difference Test”, which was used on the significant ANOVA values from Tables 8 & 9. This was conducted on all collected data with respect to the phosphite variable, since there was a significant response detected in each group. The group labeled “total per cm” will be conducted with consideration for the interaction detected between Fungus and Phosphite variables.

Tukey’s Honestly Significant Difference Test

Tukey’s Honestly Significant Difference Test (Tukey’s HSD) is a multiple-comparisons technique (Zar and others, 1999). It was performed using the software platform R with the assistance of the package title multcomp (Hothorn et al., 2014; Team and others, 2012). Tukey’s HSD was conducted on all collected data sets against the “Phosphite” factor, because all factors showed a significant response from the quantity of phosphite applied. The only collection that showed a difference in the inoculated vs. the non-inoculated [with ectomycorrhizal fungi spores] groups was at the first height data collection. No multiple comparisons were conducted on these significant results because they contain only two options and a significant difference indicates that the two options are significantly different. See the Boxplots 1-8 in the results section for the Tukey’s HSD results.

Seedling Survival

Although the experimental design did not allow for a proper statistical analysis to test significant survival differences between treatments there were striking apparent visible differences

(Figure 3). These visual differences encouraged further statistical analyses to quantify their significances. The data are essentially binary counts (plant lived =1, plant died = 0) to produce the values observed in Tables 4-6 in the results section. Binary counts cannot simply be run through an ANOVA to determine statistical significance. So another approach was taken was to perform binomial multiple comparisons using the Marascuilo Procedure.

Marascuilo Procedure

This procedure compares the proportions among all pairwise groups and creates a critical range value that is then compared to the absolute difference in proportions between the pair. If the critical range value is lower than the actual difference then the pair is significantly different. It may help to think of this as:

$$X_1 - X_2 = \text{Absolute Difference,}$$

where X is the observed proportions being compared. This models the actual observed difference between a pair of proportions.

$$x \pm \text{Critical Range,}$$

where x is one of the proportions and the critical range is $100(1-\alpha)\%$ confidence interval, where $1-\alpha$ is the confidence coefficient for the proportion. The easiest way to determine this is by subtracting the critical range from the absolute difference, a positive value indicates a significant response. That is if,

$$\text{Absolute Difference} - \text{Critical Range,}$$

is positive then that would indicate significant response. Values from this calculation should be between -1 and 1 after complete analysis. Table 3 contains the values from the Marascuilo Procedure.

Analysis of Variance

Analysis of variance (ANOVA) was utilized to determine if the visual difference in seedling survival between treatments in the photos in Figure 3 was statistically significant. In the base format in Table 5, the number of observations per treatment was one. One observation per treatment makes ANOVA impossible, so the data had to be modified in order to have variation that could be compared.

Data Reconstruction

Observations were artificially created using a strict set of rules. Each treatment contained 20 plants and each plant was individually labeled 1-20. This was then turned into multiple observations by utilizing plants 1-5 as observation 1, plants 6-10 as observation 2, plants 11-15 as observation 3 and finally plants 16-20 as observation 4. Survivors were labeled 1 and deceased were labeled a 0 and the values were summed to generate the observational values. The result is that each treatment had 4 observations and each observation could have a value of 0 to 5.



Figure 3. Apparent visual differences in seedling size and survival across all treatments. The group on the left in each photo was inoculated with spores of *Pisolithus tinctorius*.

Shapiro-Wilkes and Transformations

These groups were tested for the ANOVA assumptions, but the data failed normality assumption under the Shapiro-Wilkes test. Transformations and alternate numbers of observations per treatment were attempted to resolve the violations of assumptions. The problem is that as the number of observations decreases as the variance decreases and the data become less normally distributed. Conversely, as the number of observations increase the data become more normally distributed but variance increases. This makes it very difficult with a small pool of data to meet the assumptions for normality.

Heteroscedasticity-Corrected Covariance Matrices

A final parametric attempt was made using Heteroscedasticity-Corrected Covariance Matrices (HCCME), which allowed the homogeneity of variance assumption to be ignored. This was effective at making the data usable but it was ineffective at generating a data set which could resolve the differences between phosphite treatments and met the normality assumption. The test was able to detect (just like the Marascuilo Procedure) a significant difference between the two treatments under the ectomycorrhizal spore applications.

Friedman's Test

The survivors data set was attacked non-parametrically with ANOVA. The appropriate test was determined to be Friedman's test. Friedman's test is a non-parametric two-factor analysis of variance.

Non-Parametric Analysis of Variance (NANOVA) Bootstrap Analysis

Further analysis was pursued with Non-Parametric Analysis of Variance (NANOVA) as described in Zhou and Wong (2011). The test was conducted utilizing the R library TANOVA which was produced by Zhou and Xu (2010) at Wing Wong's lab in the Stanford Genome Technology Center.

CHAPTER III

RESULTS

Seedling Survival

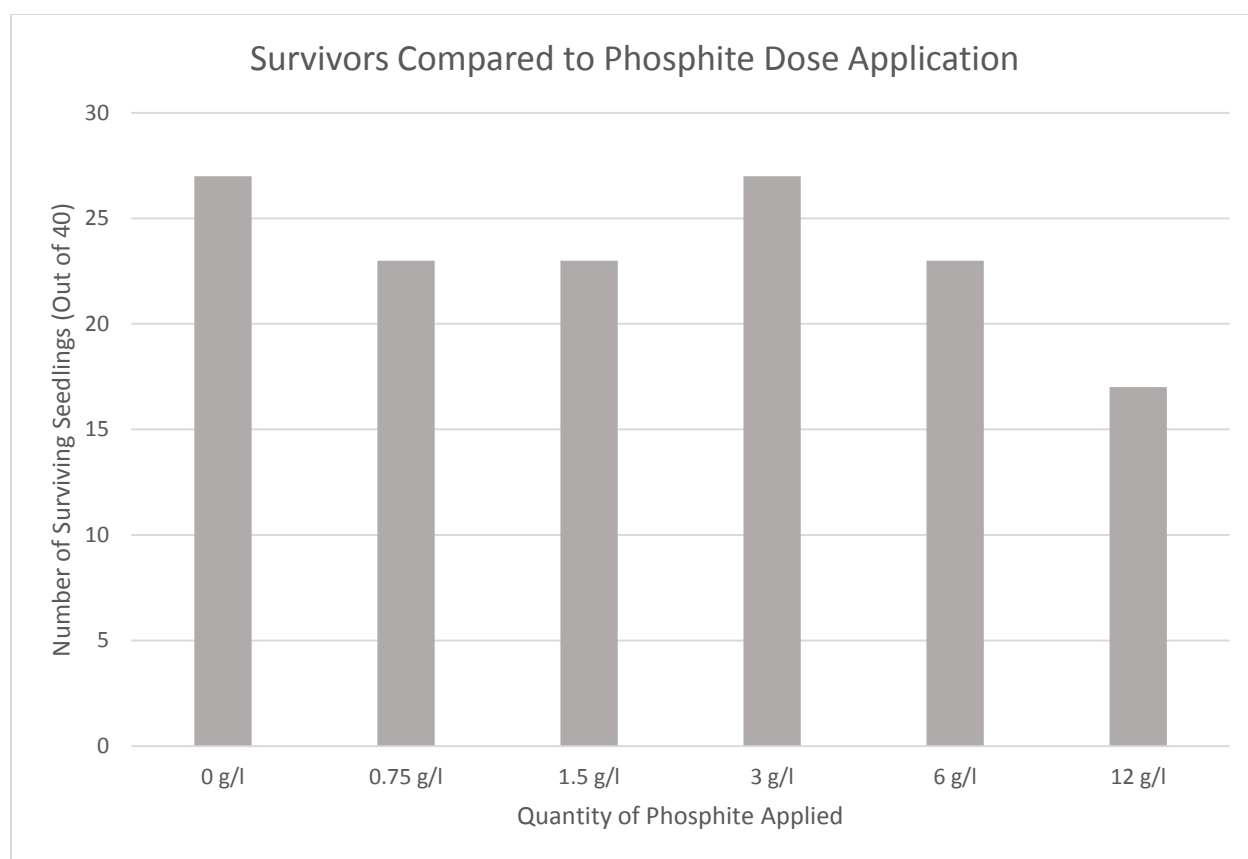


Figure 4. The effect of phosphite dose on seedling survival.

As can be seen in Figure 3, there are rather obvious differences which can be observed visually between the various treatments. The number of surviving trees per treatment is presented in Figures 4 & 5. The two treatments along the phosphite which appeared to have the best survival rates were 0.0 g/l and 3.0 g/l. The 12.0 g/l treatment had the worst survival.

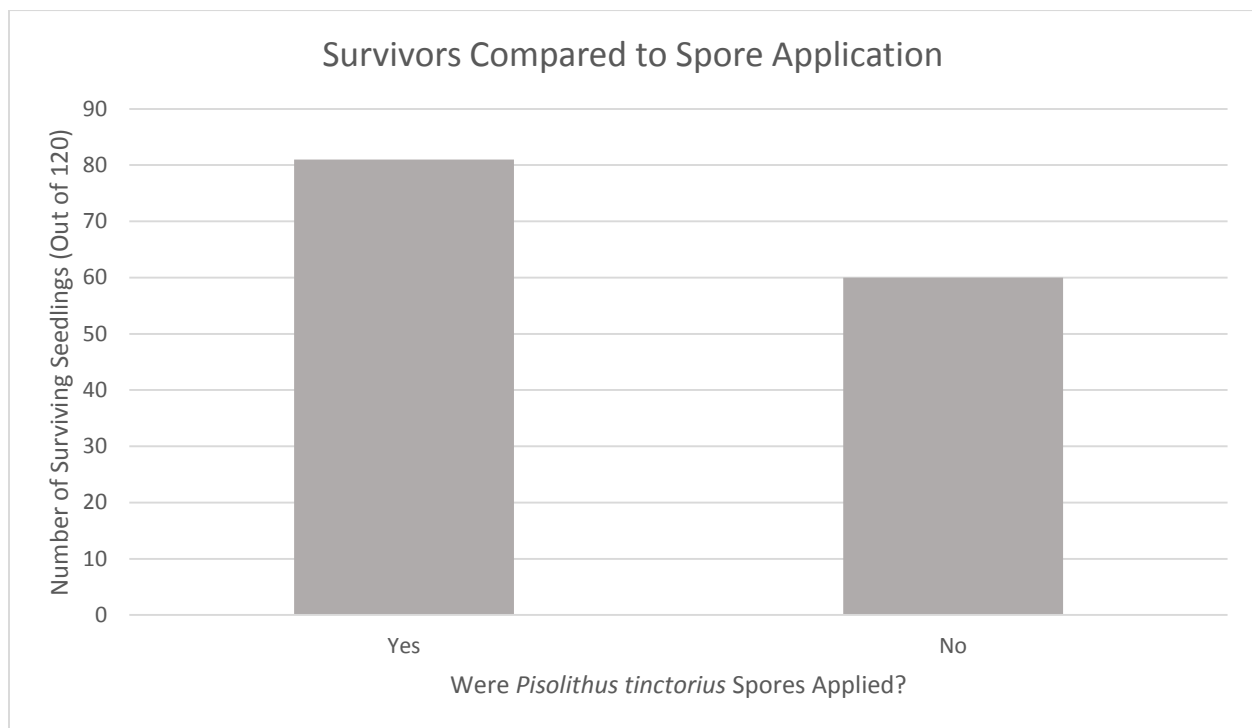


Figure 5. The effect of mycorrhizal inoculation on seedling survival.

Means for seedling height, root collar diameter, length of second-order roots sampled, the number of third-order root branches with ectomycorrhizas, third-order root branches without ectomycorrhizas, the total number of third-order root branches, ectomycorrhizal third-order roots per centimeter of second-order root, non-ectomycorrhizal third-order roots per centimeter of

second order root, the total number of third-order roots per centimeter of second-order root, and survival counts for all 12 treatments are presented in Tables 4-6. Seedlings which were replanted were not included in the Tables and thus the replant data were not analyzed. Each treatment group began with 20 seeds. The final number of plants at the end of the experiment is listed as 'survivors'.

Seedling Survival Analysis

The number of surviving trees in each of the phosphite treatment groups is presented in Figure 3 and Tables 1 & 4-6. The number of surviving seedlings varied by phosphite treatment. Significant difference in seedling survival, between treatments that received *P. tinctorius* spore inoculations and those that did not. The collected data were tested with the Marascuilo Procedure, Friedman's Test and Non-Parametric Analysis of Variance (NANOVA) Bootstrap Analysis.

Marascuilo Procedure

The results of the Marascuilo procedure (utilized in the attempt to determine if the number of survivors between treatment phosphite levels was significant) are presented in Table 3, where a positive value indicates a significant difference. Unfortunately, no significant difference was detected between phosphite treatment levels. The number of surviving trees in each of the mycorrhizal inoculation treatment groups is presented in Figure 4. There was a significant effect detected between the treatments which received spore inoculations and the treatments that did not.

Table 3. Marascuilo Procedure End Values for Seedling Survival(a positive value indicates that there is a significant difference).

Treatment	Absolute Difference - Critical Range											
	A	B	C	D	E	F	G	H	I	J	K	L
A		-0.427296	-0.46742	-0.53468	-0.50331	-0.38315	-0.38315	-0.38315	-0.38315	-0.4273	-0.28315	-0.103313766
B	-0.427296		-0.62816	-0.4985	-0.56535	-0.64252	-0.64252	-0.64252	-0.64252	-0.68717	-0.54252	-0.365347653
C	-0.467416	-0.628162		-0.53895	-0.60604	-0.58358	-0.58358	-0.58358	-0.58358	-0.62816	-0.48358	-0.306041634
D	-0.534682	-0.498501	-0.53895		-0.57533	-0.45416	-0.45416	-0.45416	-0.45416	-0.4985	-0.35416	-0.175332412
E	-0.503314	-0.565348	-0.60604	-0.57533		-0.52087	-0.52087	-0.52087	-0.52087	-0.56535	-0.42087	-0.242786901
F	-0.383149	-0.642516	-0.58358	-0.45416	-0.52087		-0.69782	-0.69782	-0.69782	-0.64252	-0.59782	-0.420869305
G	-0.383149	-0.642516	-0.58358	-0.45416	-0.52087	-0.69782		-0.69782	-0.69782	-0.64252	-0.59782	-0.420869305
H	-0.383149	-0.642516	-0.58358	-0.45416	-0.52087	-0.69782	-0.69782		-0.69782	-0.64252	-0.59782	-0.420869305
I	-0.383149	-0.642516	-0.58358	-0.45416	-0.52087	-0.69782	-0.69782	-0.69782		-0.64252	-0.59782	-0.420869305
J	-0.427296	-0.687168	-0.62816	-0.4985	-0.56535	-0.64252	-0.64252	-0.64252	-0.64252		-0.54252	-0.365347653
K	-0.283149	-0.542516	-0.48358	-0.35416	-0.42087	-0.59782	-0.59782	-0.59782	-0.59782	-0.54252		-0.520869305
L	-0.103314	-0.365348	-0.30604	-0.17533	-0.24279	-0.42087	-0.42087	-0.42087	-0.42087	-0.36535	-0.52087	
Phosphite	0	0.75	1.5	3	6	12						
0		-0.25825	-0.28156	-0.34846	-0.25825	-0.10825						
0.75	-0.25825		-0.34113	-0.25825	-0.36778	-0.21778						
1.5	-0.281556	-0.341129		-0.28156	-0.34113	-0.19113						
3	-0.34846	-0.25825	-0.28156		-0.25825	-0.10825						
6	-0.25825	-0.367779	-0.34113	-0.25825		-0.21778						
12	-0.10825	-0.217779	-0.19113	-0.10825	-0.21778							
Ectomycorrhiza	Spores	No Spores										
Spores		0.0524279										
No Spores	0.0524279											

Friedman's Test

Results of Friedman's test (survivors data were compared across all 12 treatments), yielded no significant differences (p-value = 0.2416). The different levels of phosphite again did not show statistically significant differences (p-value = 0.21). When comparing inoculation and non-inoculation of ectomycorrhizal spores, the test detected a highly significant difference (p-value = 0.00729). These results confirmed the findings of the Marascuilo procedure.

Non-Parametric Analysis of Variance (NANOVA) Bootstrap Analysis

Results (p values) of a bootstrapped NANOVA analysis (a final attempt to find significant results along the phosphite gradient), are presented in Table 10. The NANOVA was run in R at 10,000, 100,000, and 1,000,000 bootstrap samples on Fungus*Phosphite interaction, Fungus + Phosphite, and Fungus and Phosphite main effects. The NANOVA showed that the observed differences in survival between phosphite treatment groups were not statistically significant.

Tree Height

Generally speaking, the heights of trees were taller in groups that received *Pisolithus tinctorius* spores at the onset of the experiment and in the treatments which received either 0.0 g/l of phosphite or an amount of phosphite within the manufacturer's recommendation. The statistically significant differences are shown in Tables 7-9 and Boxplots 3-5. Plants did not increase in height between 30 September 2012 and 10 November 2012. The 12.0 g/l group in Boxplot 3 has outliers on either side of the data set and a decreased variance, a result of a less complete data set ($n = 17$) and the high variance of the entire data set. This dissappeared as time progressed for the latter samples, as depicted in Boxplots 4 & 5.

The first height data produced similar results to the first root collar data. The 12.0 g/l group was statistically the shortest while 1.5 g/l was the tallest. The 0.0 g/l phosphite group was a little closer to 1.5 g/l and distinctly different from 12.0 g/l as well. The remaining three groups (6.0, 3.0 and 0.75) produced results that showed little difference between the 0.0 and 12.0 g/l groups. Based on this data set and the manufacturers' recommended dosing, there was no benefit seen utilizing

over 3.0 g/l. At 3.0 g/l and below, the two best options were 0.0 g/l and 1.5 g/l, there were generally supported by both root collar data sets.

Table 4. Means for seedling height, root collar diameter, mycorrhizal quantification, and survival counts for all 12 treatments.

					Tree Height In mm			Root Collar Diameter In mm									
					8/8/2012	9/30/2012	11/10/2012	9/30/2012	11/17/2012								
	Phosphite Concentration Applied	Group	Plants	Survivors						Length Of Root Sample In cm	Root Branch With Ecto	Root Branch Without Ecto	Total Branch	Ecto Per cm	No Ecto Per cm	Total Per cm	
Spores n=120 survivor= 81	0	A	20	16	563	876	871	12.4	14.2	43	50	114	164	1.16	2.64	3.80	
	0.75	B	20	12	503	782	754	10.8	12.5	38	85	141	226	2.31	3.70	6.01	
	1.5	C	20	13	666	1007	978	13.6	15.0	58	81	179	260	1.38	3.00	4.37	
	3	D	20	15	531	968	955	12.9	15.1	45	63	169	232	1.44	3.75	5.19	
	6	E	20	14	488	851	885	11.0	14.3	36	30	195	225	0.96	5.34	6.31	
	12	F	20	11	354	503	552	7.8	10.5	39	25	179	204	0.80	4.53	5.33	
no spores n=120 survivor= 60	0	G	20	11	496	669	647	12.2	16.0	51	88	202	290	1.76	4.00	5.76	
	0.75	H	20	11	480	741	735	11.2	13.7	51	98	151	249	1.90	3.03	4.93	
	1.5	I	20	11	608	1006	1001	13.4	14.1	48	43	207	250	0.90	4.41	5.31	
	3	J	20	12	434	834	834	9.8	12.4	48	78	201	279	1.60	4.15	5.75	
	6	K	20	9	397	675	672	8.8	11.8	32	26	159	185	0.86	5.30	6.16	
	12	L	20	6	381	653	648	10.4	12.4	60	9	199	208	0.19	3.51	3.71	

Height at the second measurement time, when analyzed with Tukey's HSD test showed that the 1.5 and 3.0 g/l groups contained significantly taller trees compared to the 12.0 g/l group. However, the test was unable to determine in which group the remaining three dose groups belong. Visually examining the data, it appears 1.5 and 3.0 are the best groups the 0.0, 0.75 and 6.0 groups are somewhat lower, though not statistically, and the 12.0 g/l group is at the very bottom. A slightly

larger sample size may very well have statistically placed 1.5 and 3.0 g/l in an undisputed top group. The third height group experienced very little growth. The Tukey's HSD test had practically identical results and warrants no separate examination.

Table 5. Means for seedling height, root collar diameter, mycorrhizal quantification, and survival counts for all 6 phosphite treatments.

Phosphite Concentration Applied	Group	Plants	Survivors	Tree Height In mm			Root Collar Diameter In mm		Length Of Root Sample In cm	Root Branch With Ecto	Root Branch Without Ecto	Total Branch	Ecto Per cm	No Ecto Per cm	Total Per cm
				8/8/2012	9/30/2012	11/10/2012	9/30/2012	11/17/2012							
0	A,G	40	27	536	792	780	12.3	14.9	46	66	150	215	1.41	3.19	4.60
0.75	B,H	40	23	492	762	745	11.0	13.1	44	91	146	237	2.11	3.38	5.49
1.5	C,I	40	24	639	1007	989	13.5	14.6	53	64	192	256	1.16	3.64	4.80
3	D,J	40	27	488	908	901	11.5	13.9	47	69	183	253	1.51	3.93	5.44
6	E,K	40	23	453	782	802	10.1	13.3	35	28	181	210	0.92	5.33	6.25
12	F,L	40	17	363	556	586	8.7	11.2	46	19	186	206	0.58	4.17	4.75

Root Collar Diameter

Unlike the height measurements the root collar diameter did increase by 10-25% between 30 September 2012 and 17 November 2012. Similar to height, the measurements of the root collar diameters were greatest at 0.0 g/l and within the manufacturer's recommendation. Statistically significant results are marked in Tables 7-9 and Boxplots 1 & 2. In Boxplot 1 & 2, there are some outliers on the 3.0 g/l, 1.5 g/l and the 0.0 g/l measurements from a statistical standpoint. These

outlier can be ignored in regards to the normal distribution assumption and the data still considered to meet normality assumption due to sufficiently high sample size (n).

Table 6. Means for seedling height, root collar diameter, mycorrhizal quantification, and survival counts for both mycorrhizal inoculation treatments.

				Tree Height In mm			Root Collar Diameter In mm								
	Group	Plants	Survivors	8/8/2012	9/30/2012	11/10/2012	9/30/2012	11/17/2012	Length Of Root Sample In cm	Root Branch With Ecto	Root Branch Without Ecto	Total Branch	Ecto Per cm	No Ecto Per cm	Total Per cm
Spores	A-F	120	81	523	845	845	11.6	13.7	43	56	161	217	1.33	3.78	5.12
No Spores	G-L	120	60	475	776	769	11.1	13.5	48	62	187	249	1.30	4.07	5.38

Ectomycorrhizal Quantification

Results of ectomycorrhizal quantifications for all treatments are presented in Tables 4-6. The tables include the results of measurements of the length of the second-order roots sampled in cm, the number of third-order roots with ectomycorrhizas, the number of third-order roots without ectomycorrhizas and the total number of third-order roots. The tables also include calculated values for the number of ectomycorrhizal third-order roots per centimeter of second order root, the number of non-ectomycorrhizal third-order roots per centimeter of second order root, and the total number of third-order roots per centimeter of second order root. ANOVAs were conducted on the calculated values and the results are listed in Tables 7-9. The phosphite factor was examined with multiple comparisons and those results can be seen in Boxplots 6-8. In the

boxplots there are a few outliers and in Boxplot 8 the 0.0 g/l treatment level appears to be skewed. These were considered non-significant violations of ANOVA assumptions because of the central limit theorem.

In the ectomycorrhizal tertiary branches per cm of linear secondary root length category, the Tukey's HSD test results indicated that 0.0, 0.75 and 3.0 g/l were equal for the most mycorrhizal branches per cm. The 0.75 g/l group was in the undisputed best category. The 0.0 and 3.0 g/l were members of a mixed category that contained 1.5 g/l and 6.0 g/l groups. The 1.5 g/l and 6.0 g/l groups shared category space with the 12.0 g/l group. 12.0 g/l was definitively the ranking with the least ectomycorrhizal branches. Visual examination of the boxplots suggests three groups - 0.75 then 0.0, 1.5, 3.0 and finally 6.0, 12.0 g/l. As in the previous data group, a slight increase in sample size might have increased the power enough to gain clear differentiation between these middle groups.

The data that examined tertiary branches/cm without ectomycorrhizal tips only differentiated two groups. The first group contained the 0.0, 0.75, 1.5 and 3.0 g/l dose concentrations and had significantly the least number of non-ectomycorrhizal tertiary root branches. The 6.0 g/l group had the greatest number of non-ectomycorrhizal tertiary root branches. Finally, the 12.0 g/l was lacking enough power to differentiate between the two groups, most likely due to high mortality within this group.

Total branchiness was the final category examined. The Tukey's HSD test placed 6.0 g/l in the most branchy second order lateral roots group - the group with the most tertiary branches emanating from the secondary root structure. The 0.0 g/l group was the least branchy and the other four groups showed inconclusive results. This whole category revealed very little difference and lots of variation.

Tukey's Honestly Significant Difference Test

In the Tukey's HSD test groups (Boxplots 1-8) there was significant overlap of the underlying distributions between phosphite treatment levels. In the Tukey's HSD test groups (Boxplots 1-8) there was significant overlap of the underlying distributions between phosphite treatment levels. This was a result of the large variance associated with the data in the different responses. In many instances this overlap was non-problematic due to a very sizable n value. The result is that the boxplot comparisons have significant overlap in the images. This was concerning and the statistics were thoroughly rechecked to ensure correctness. The determination is that the overlap of these boxplots is a direct result of the overlap of the wide variation present in the underlying distributions and that these boxplots do indeed model the data correctly. The median (indicated by the dark horizontal lines in the boxplots) are significantly different where they are marked as different by the grouping at the top of each boxplot. These differences were detectable due to the increased sample size and could very easily have not been detected with an inadequate sample.

Table 7. Key for Tables 8 & 9

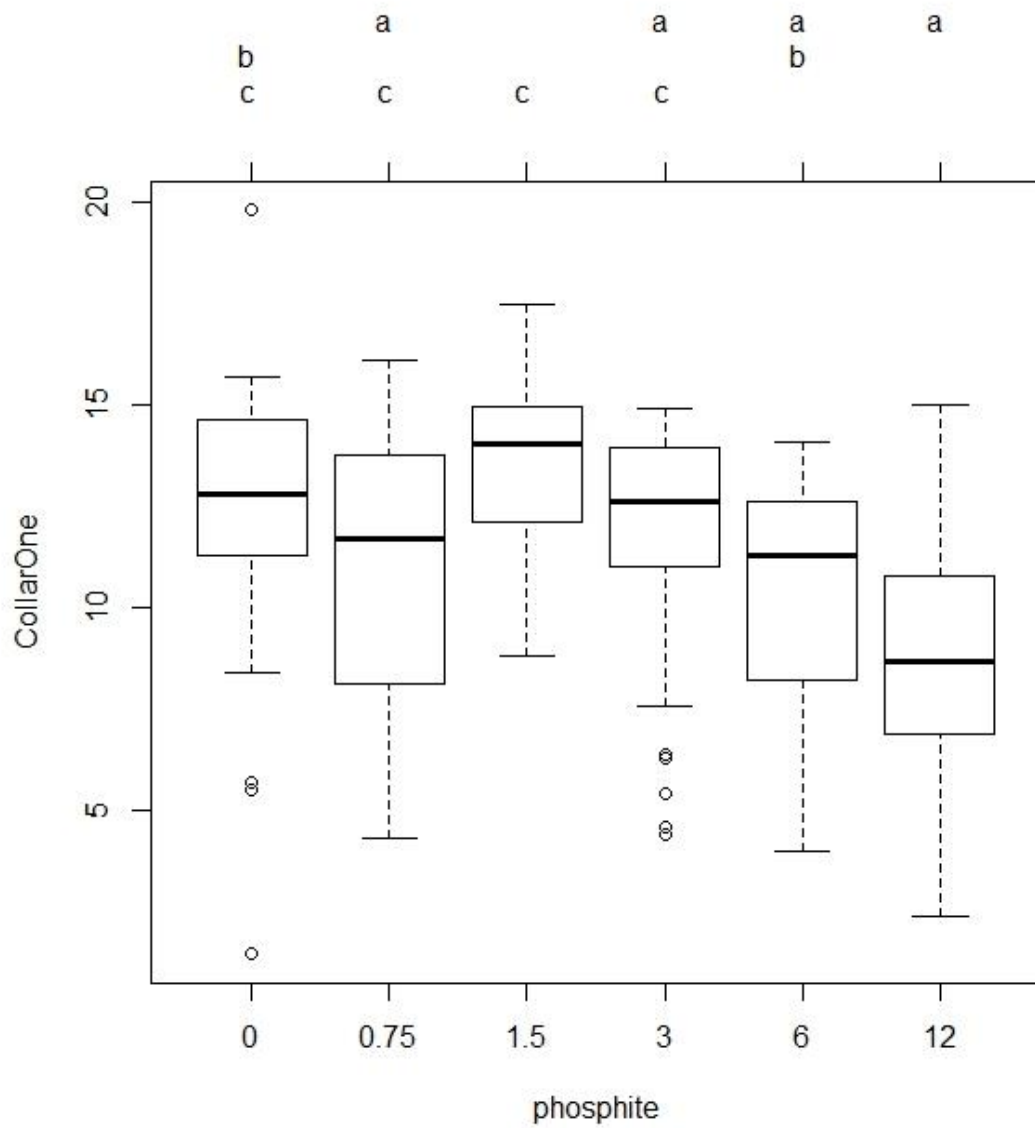
	Not Significant	90% Significant	95% Significant	99% Significant	99.5% Significant	99.9% Significant
Key	$0.1 < X < 1.0$	$0.05 < X < 0.1$	$0.01 < X < 0.05$	$0.005 < X < 0.01$	$0.001 < X < 0.005$	$0.0 < X < 0.001$

Table 8. ANOVA p-values for all responses for both main factors and interactions.

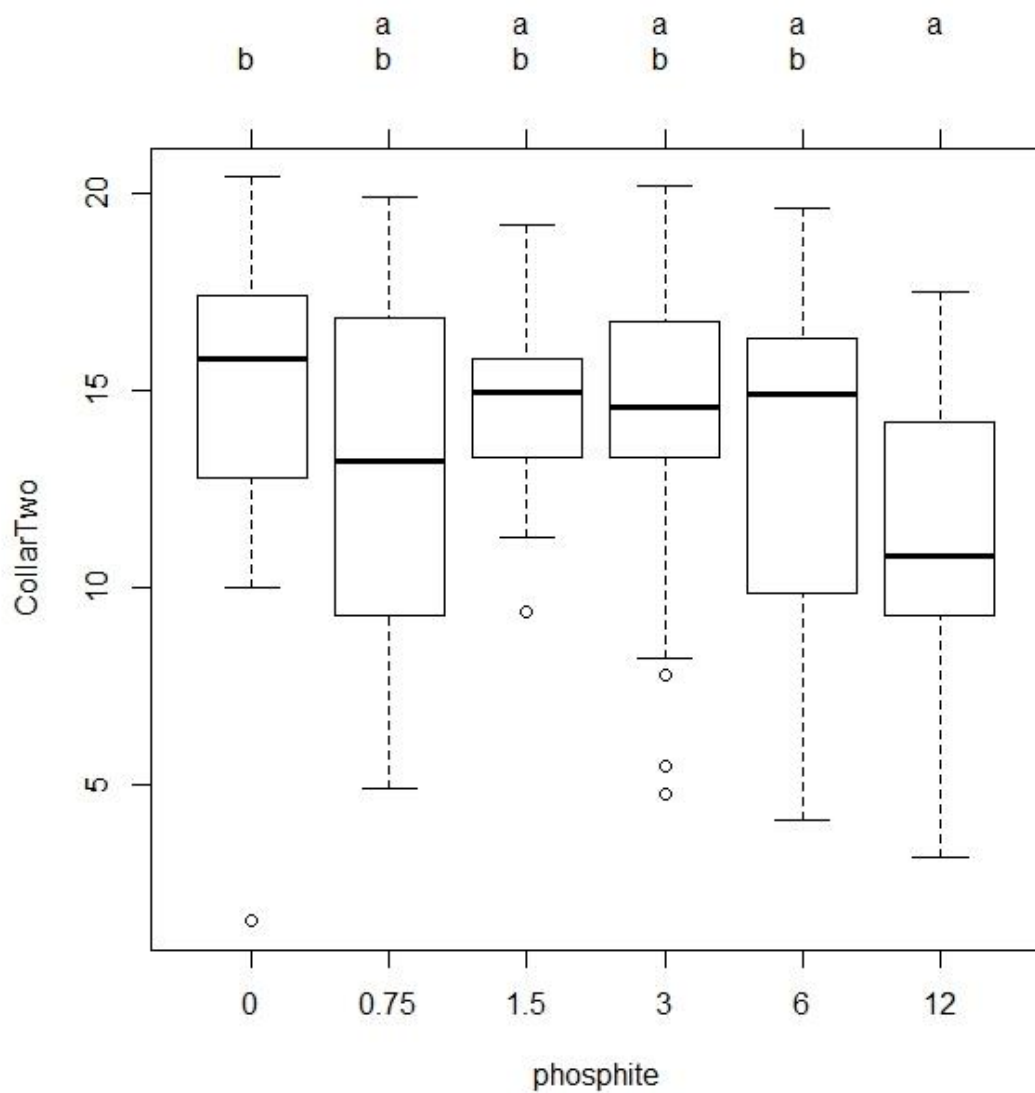
ANOVA p-values (Phosphite * Fungus and Fungus * Phosphite)				
		Fungus		
		Fungus	Phosphite	Fungus*Phosphite
Measurements	Height 8/8/2012	0.0888	0.0000274	0.8656
	Height 9/30/2012	0.2258	0.00135	0.57759
	Height 11/10/2012	0.1769	0.00405	0.5364
	Collar 9/30/2012	0.365	0.000123	0.094
	Collar 11/17/2012	0.7452	0.0448	0.1492
	Ecto per cm	0.83	0.00000159	0.155
	No Ecto per cm	0.2798	0.000107	0.0557
	Total per cm	0.3925	0.0138	0.0115

Table 9. ANOVA p-values for all responses for both main factors and ignoring interactions.

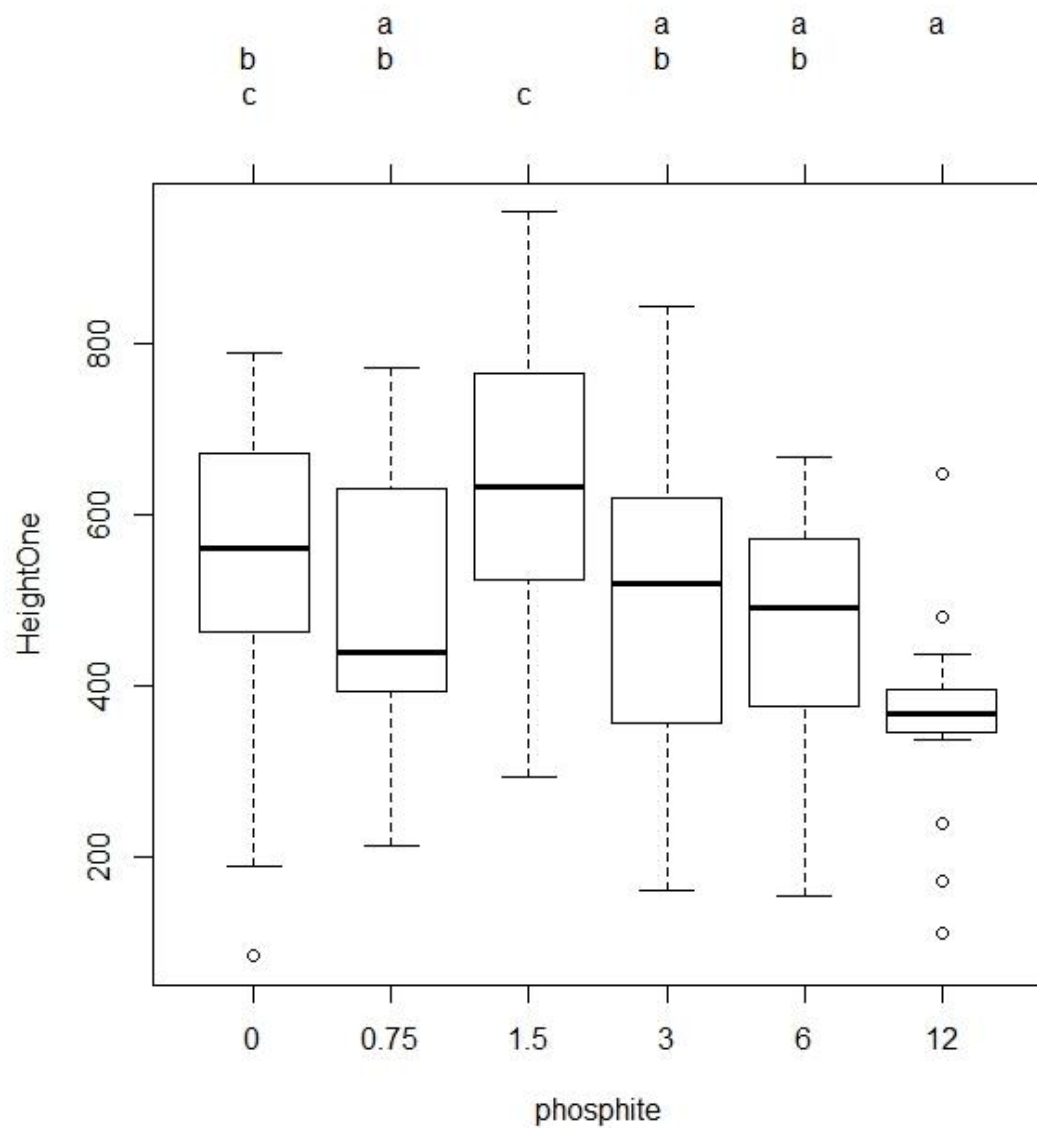
ANOVA p-values (Phosphite + Fungus)			
		Factors	
		Fungus + Phosphite	
		Fungus	Phosphite
Measurements	Height 8/8/2012	0.085	0.0000198
	Height 9/30/2012	0.22368	0.00123
	Height 11/10/2012	0.175	0.00382
	Collar 9/30/2012	0.372767	0.000168
	Collar 11/17/2012	0.7481	0.0493
	Ecto per cm	0.832	2.07E-06
	No Ecto per cm	0.290449	0.000163
	Total per cm	0.4101	0.0205



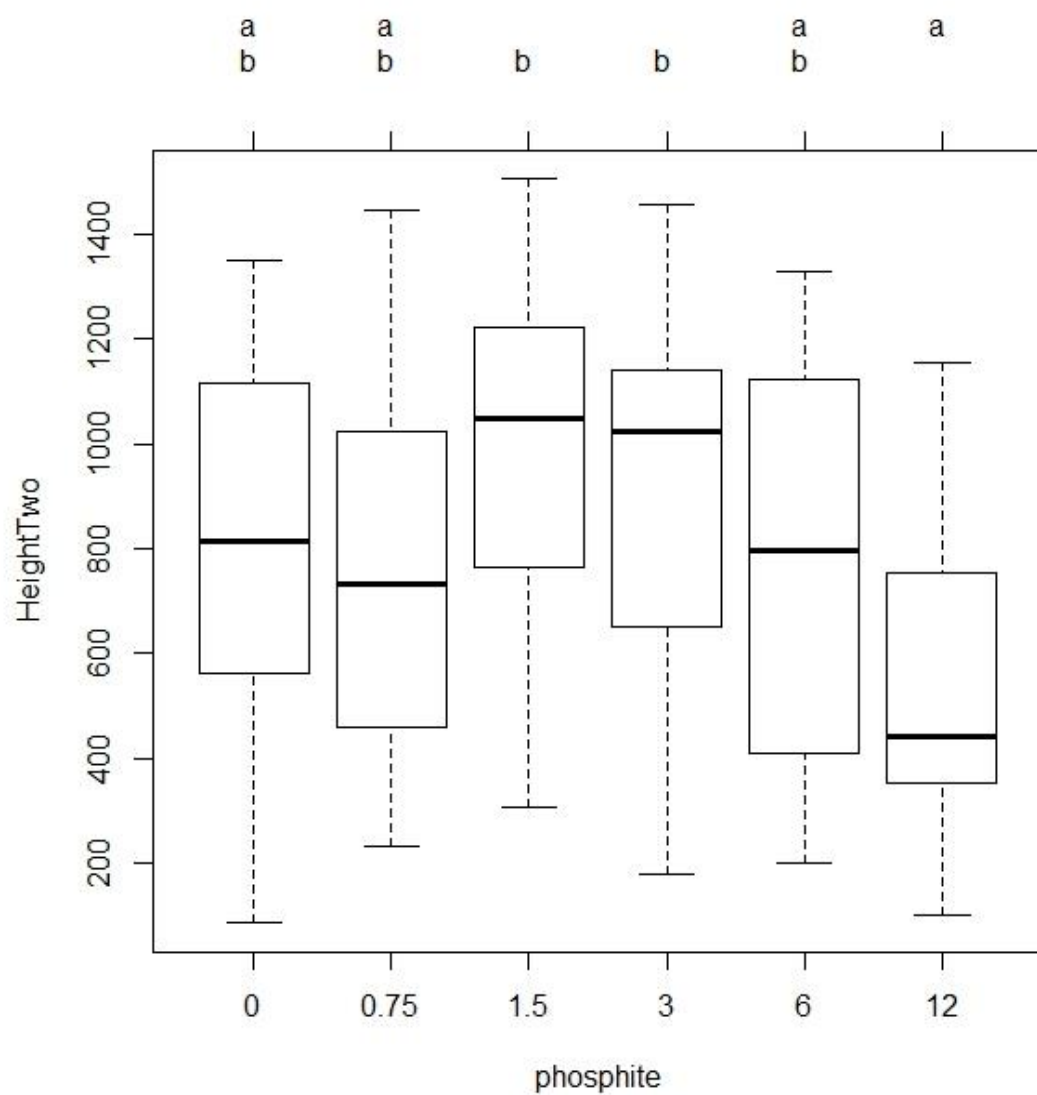
Boxplot 1. Root collar diameter data 30 September 2012 multiple comparisons across phosphite treatments.



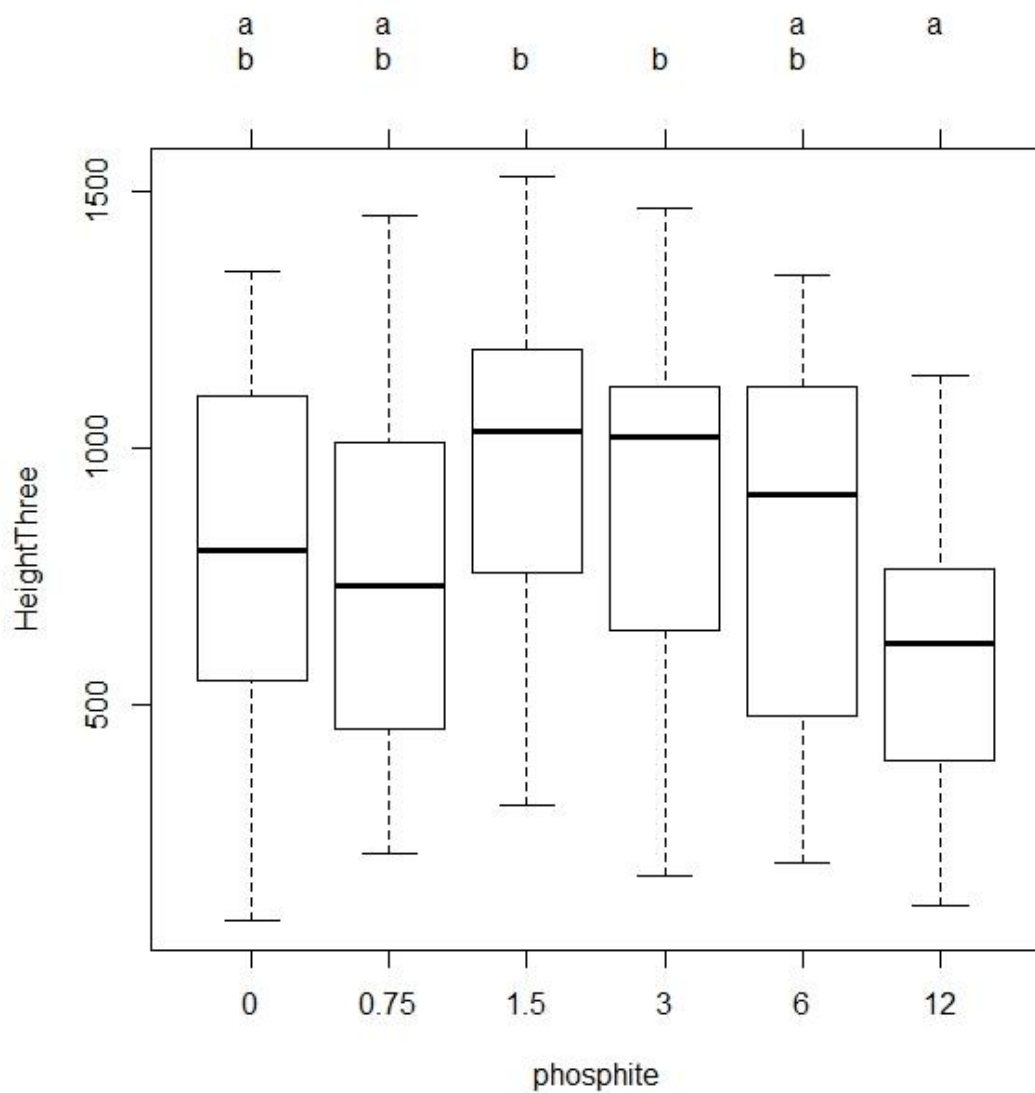
Boxplot 2. Root collar diameter data 17 November 2012 multiple comparisons across phosphite treatments.



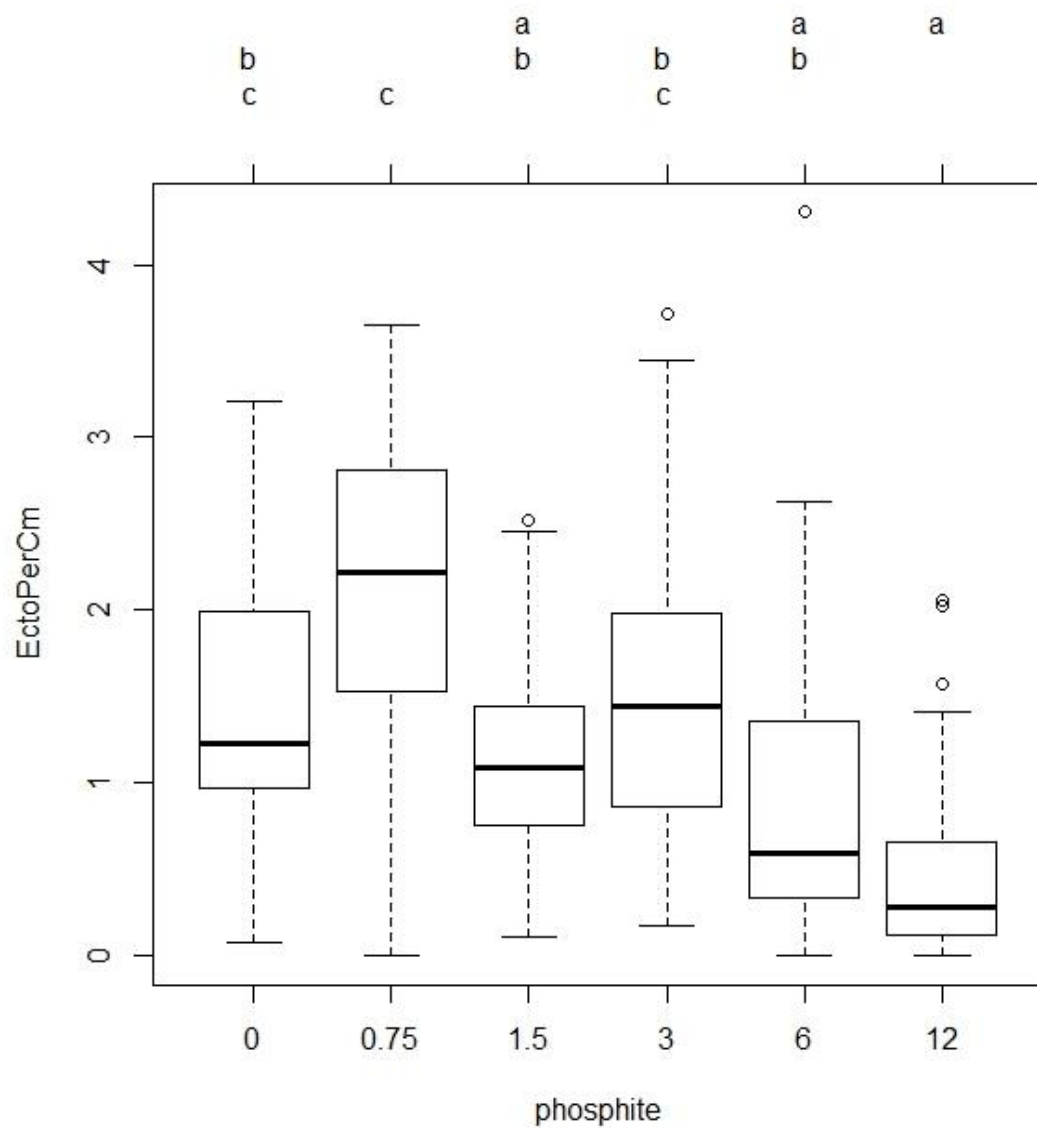
Boxplot 3. Height data from 8 August 2012 multiple comparisons across phosphite treatments.



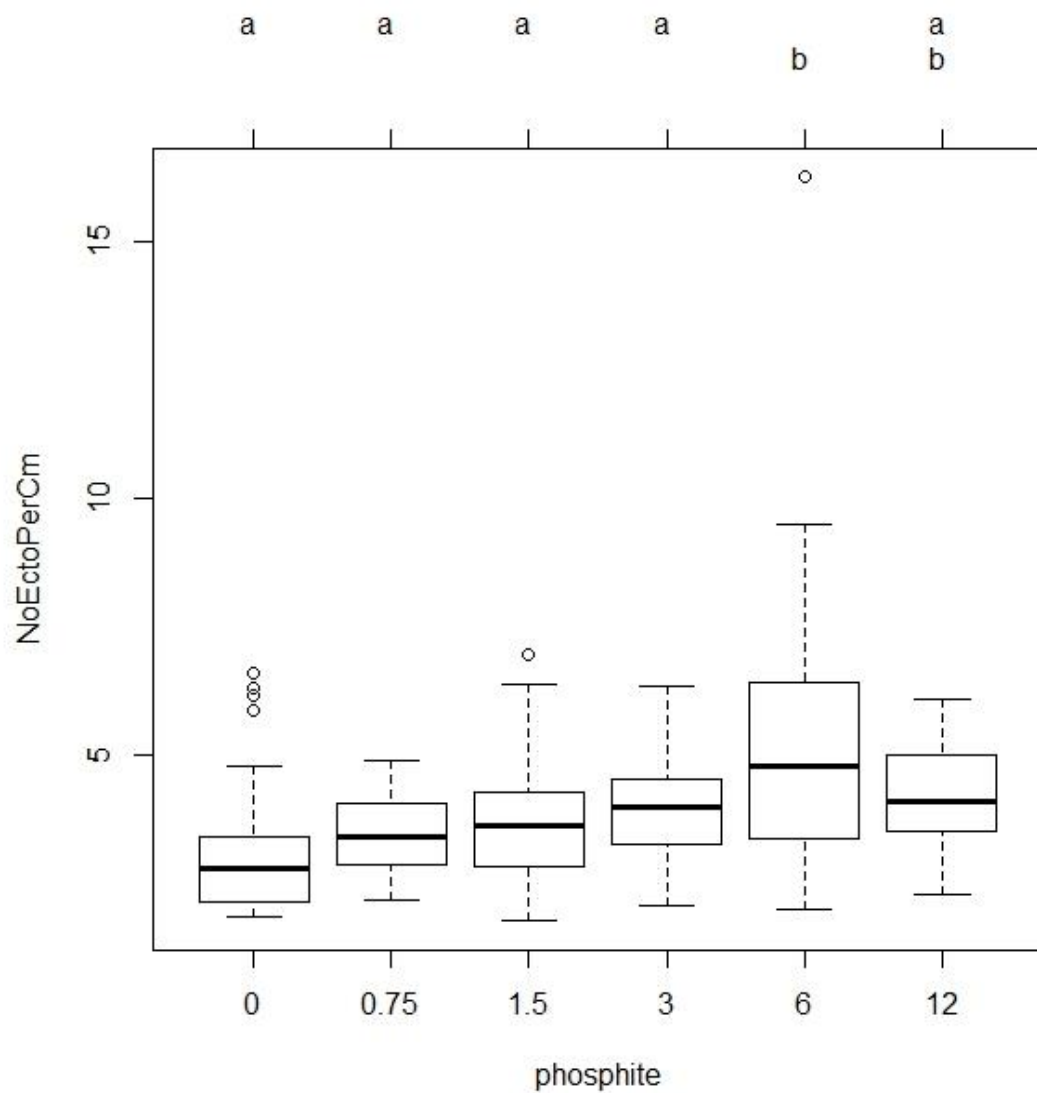
Boxplot 4. Height data from 30 September 2012 multiple comparisons across phosphite treatments.



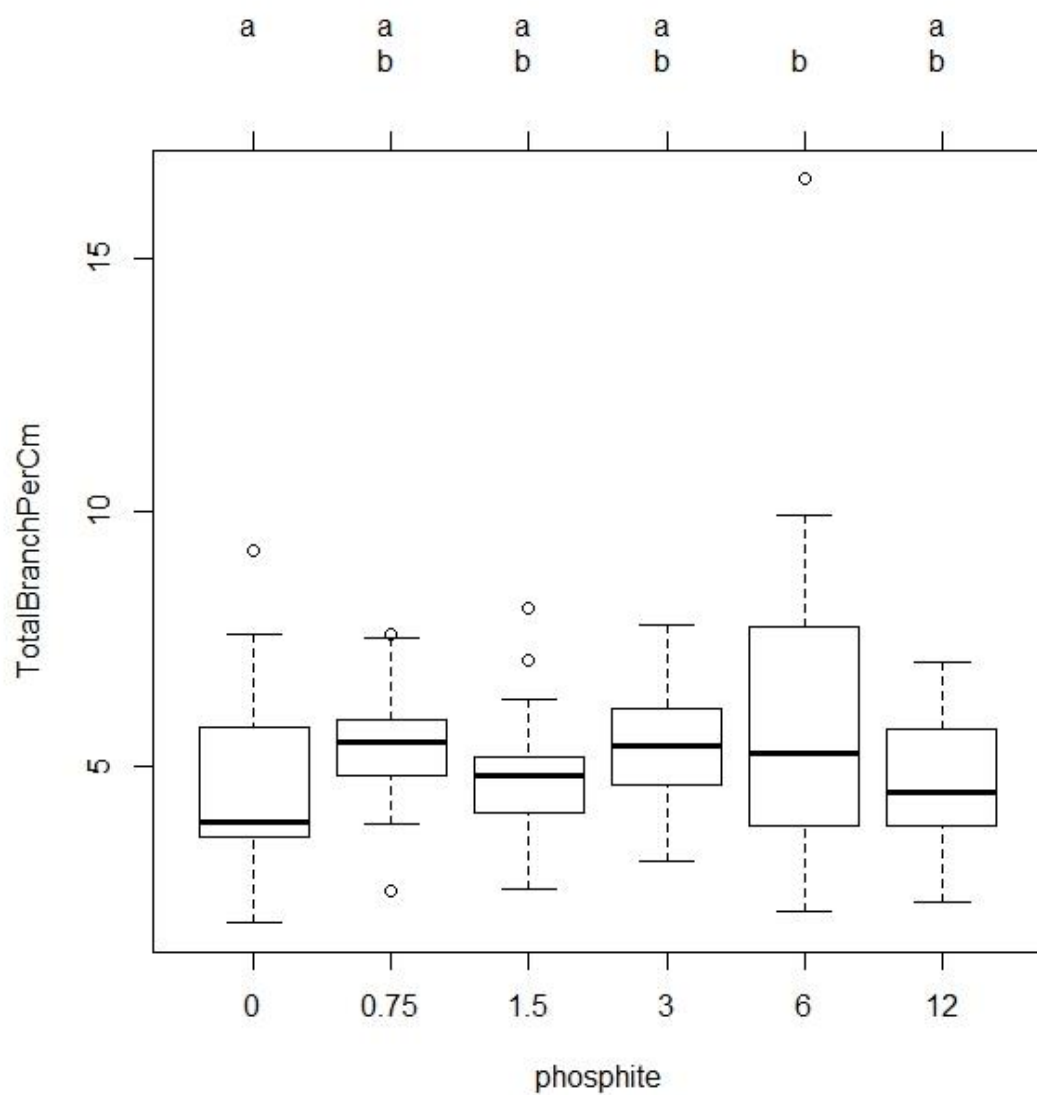
Boxplot 5. Height data from 10 November 2012 multiple comparisons across phosphite treatments.



Boxplot 6. Ectomycorrhizal branches 17 November 2012 multiple comparisons across phosphite treatments.



Boxplot 7. Non-Ectomycorrhizal branches 17 November 2012 multiple comparisons across phosphite treatments.



Boxplot 8. Total branches 17 November 2012 multiple comparisons across phosphite treatments.

Table 10. Non-parametric Analysis of Variance (NANOVA) for Seedling Survival (p-values for three different bootstrap resample sizes).

	Resample Size		
	10,000	100,000	1,000,000
Interaction	0.6877	0.68889	0.687889
Additive effect	0.4871	0.48044	0.480175
Phosphite	0.2186	0.21625	0.216631
Spore inoculation	0.6293	0.62544	0.622236

Root Scans

Results of the visual examinations of scanned root system samples show statistically significant differences between a high phosphite application treatments and lower phosphite application treatments. Visual differences can be seen in Figures 6 & 7. Figure 6 shows an example of one of the plants that received lower dosage of phosphite while Figure 7 shows an example of one of the plants that received a higher dose of phosphite. As the concentration of phosphite increases the development of the root system seems to be reduced.

Comparison of Tukey's Honestly Significant Difference Test

Following examination of the individual Tukey's HSD tests, the results between multiple comparisons groups were compared to each other. When the root collar diameter and the sapling height data were considered together, the phosphite treatment level that produced the best results was 1.5 g/l. This was determined by examining the groupings in the Tukey's HSD test. If a

particular group was in the best and/or worst group, then it received a point for each row accordingly.

Table 11. A comparison of the Tukey's HSD boxplots for the height and root collar diameter responses; tallies of phosphite treatments that were good or bad (a higher value is better).

	Root Collar Diameter + Sapling Height Results					
	0.0 g/l	0.75 g/l	1.5 g/l	3.0 g/l	6.0 g/l	12.0 g/l
Best Group Tukey's HSD	5	4	5	4	3	0
Worst Group Tukey's HSD	2	5	1	3	5	5
Best - Worst	3	-1	4	1	-2	-5

Using this method, the preferential order of the groups (from best to worst) is: $1.5 > 0.0 > 3.0 > 0.75 > 6.0 > 12.0$ g/l. If, instead, the third height measurement is removed (it was essentially a duplicate of the second height data) as a correction, then the modified results suggest $1.5 = 0.0 > 3.0 > 0.75 > 6.0 > 12.0$ g/l.

Table 12. A comparison of the Tukey's HSD boxplots for the height and root collar diameter responses; tallies of phosphite treatments that were good or bad (a higher value is better). The third height measurement data were excluded to remove bias.

	Root Collar Diameter + Sappling Height Results					
	0.0 g/l	0.75 g/l	1.5 g/l	3.0 g/l	6.0 g/l	12.0 g/l
Best Group Tukey's HSD	4	3	4	3	2	0
Worst Group Tukey's HSD	1	4	1	3	4	4
Best - Worst	3	-1	3	0	-2	-4

If further consideration is given to the ectomycorrhizal component and it is factored into the modified results (with the assumptions: more ectomycorrhizal branches = better, more non-ectomycorrhizal branches = worse, more total branches = better), then the results suggest $0.0 > 1.5 > 3.0 > 0.75 > 6.0 > 12.0$.

Table 13. A comparison of the Tukey's HSD boxplots for the height and root collar diameter and root responses. Tallies of phosphite treatments that were good or bad, a higher value is better.

	Root Collar Diameter + Sappling Height Results					
	0.0 g/l	0.75 g/l	1.5 g/l	3.0 g/l	6.0 g/l	12.0 g/l
Best Group Tukey's HSD	6	6	6	6	3	2
Worst Group Tukey's HSD	2	5	3	4	6	7
Best - Worst	4	1	3	2	-3	-5



Figure 6. Scan showing mycorrhizal roots and a well-developed tertiary root system.



Figure 7. Scan of root lacking mycorrhizas

CHAPTER IV

DISCUSSION

Throughout this experiment there were eight different planned responses and a ninth unplanned response examined. The close examination of these responses has indicated some general trends that were detected throughout the entire experiment. One of these trends is that the best performing treatments were those inoculated with ectomycorrhizal spores. A second major trend was that the best three groups in virtually all phosphite responses consisted of the top and bottom of the manufacturer's recommendation and no application of phosphite (0.0, 1.5, 3.0 g/l).

There were pseudosignificant results in the first height data collection. This may have resulted from the experimental choice to inoculate the seeds with spores at the start of the experiment. Consider that the first height measurement occurred shortly after moving the subjects from the inside to the outside of the greenhouse. Additionally, no action was taken to prevent natural exposure to the spores. It seems reasonable that prior to significant exposure to spores in the wild, there was potentially a significant improvement in growth with respect to height by the plants which received the ectomycorrhizal benefits. This is perhaps worthy of follow-up research if there is interest, but it may be a lower priority since the plant heights balanced out as the experiment progressed.

Unfortunately, the Marascuilo Procedure, Friedman's test, the ANOVA on the modified survivor data and the bootstrap analysis were unable to detect significant differences between individual treatments and between the concentration groups underlying the phosphite factor with

respect to plant survival. This could mean two things: either the sample size is inadequate to detect the difference or there is no difference. Looking at the photos in Figure 3 it appears that fewer plants survived at higher concentrations. This suggests that a new experiment to look at survival rates along the phosphite gradient may be warranted.

Regarding the three data sets examining the ectomycorrhizal tips on tertiary roots and the branchiness, there was no clear interpretation of the results. Increasing sample size could improve these results and allow for better interpretation. Greater benefit could be obtained with different sampling and quantification techniques. The quantification technique used in this experiment was effective at allowing more rapid quantification of the root data. This was useful as this method resulted in examination of nearly 40,000 tertiary branches. An alternate method, which involves counting individual root tips, would have resulted in the manual counting of millions of individual root tips; a sample this size would be much more time consuming. A third technique measures the length of root that is covered in ectomycorrhizae and the part that is not covered by ectomycorrhizae, converting these data into a ratio for analysis. This technique would be even more time consuming and virtually impossible to conduct manually on this scale. Ideally in a future study, the quantification of the root data could be automated. This would eliminate human error. Manual examination of 40,000 branches likely produced some error, which was evenly spread among the groups. Automation would improve accuracy and speed up the process. There was no effective way identified in this research to automate the quantification process in future experiments.

In the introduction, two questions were asked: “How do different concentrations of phosphite impact ectomycorrhizal growth in *Castanea*?” and “What dose of phosphite concentration with respect to plant and ectomycorrhizal growth is ideal for *Castanea*?” In response

to the first, it was found that higher concentrations of phosphite were mycotoxic to *P. tinctorius*, though at lower concentrations there were fewer significant differences. The second question, which is probably the more important question, is answered very clearly by the data. If root-rot in *Castanea* is a concern, then based on the literature reviewed and the results from the current experiment, the preferred protocol should be to use phosphite at the 1.5 g/l (lower end of the manufacturers recommended dose for Allude by Cleary Chemical) concentration to optimize the health of the chestnut seedling and *P. tinctorius*. The chemical should only be applied at times when infection by *P. cinnamomi* is likely. It should be noted that this experiment did not examine how effective any concentration of phosphite protected against root-rot. The protection against root-rot is very species-specific. Future research should be conducted to determine the effectiveness in treating against phytophthora root rot on a concentration gradient of phosphite. The range to examine should include 1.5 – 6.0 g/l of phosphite and the lowest concentration found to be effective at treating root-rot, within this range, should be used. This additional research in combination with the current research would produce the most effective dose for treating *Phytophthora*, while maximizing the health of *Castanea* and *P. tinctorius* and eliminating excess chemical usage, providing maximum financial efficiency for benefits achieved.

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VITA

Justin Miles Jorgensen was born 31 May 1983 in Fort Oglethorpe, Georgia. Shortly after birth, he moved to Hamilton County, Tennessee, where he still resides to the date of this publication. He attended primary and secondary school from 1987-2001 in Chattanooga, Tennessee. After graduating high school, he began college at Chattanooga State Technical Community College and graduated in 2005 with an Associates of Science. That same year he began work on his Bachelor's Degree at the University of Tennessee at Chattanooga, where he graduated with a degree in Biological Sciences. While working on his Bachelor's Degree, he took classes from and became friends with Dr. J. Hill Craddock, who interested him in chestnut trees. This friendship resulted in Miles enrolling in the Masters of Science in Environmental Science program at the University of Tennessee at Chattanooga in 2009. This manuscript is the last requirement before graduation on 13 December 2014.

Miles married his wife Mikayla Jorgensen, who was born in Vicenza, Italy in 1980, in 2005 and they had their first son Vasher in 2006, their second son Micah in 2009 and the third son, Phoenix in 2013. They currently live in a small shanty inside the town of Signal Mountain, Tennessee.